DNA TOPOISOMERASE INHIBITORS INDUCE A LOSS OF MITOCHONDRIAL DNA IN MAMMALIAN CELLS

BY

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This dissertation is dedicated to my mother, father, brother, and sister.

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KEY TO ABBREVIATIONS

ADP	Adenosine 5'-Diphosphate
ATP	Adenosine 5'-Triphosphate
AZT	3'-Azido-3'-deoxythymidine
COX	Cytochrome c Oxidase
ddC	2', 3'-Dideoxycytodine
DMEM	Delbecco's Modified Eagle's Media
DNA	Deoxyribonucleic Acid
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetretraacetic Acid
EGTA	Ethylene Glycol-bis(B-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IC_{50}	Concentration that Produces 50% Inhibiton
m-AMSA	meta-Amsacrine
MEM	Minimum Essential Media
MGBG	Methylglyoxal bis[guanylhydrazone]
mtDNA	Mitochondrial DNA
mtRNA	Mitochondrial RNA
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide, Reduced Form
o-AMSA	ortho-Amsacrine
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SDS	Lauryl Sulfate, Sodium Salt
Tris	Tris[hydroxymethyl]amino-methane
tRNA	Transfer RNA
VM-26	Teniposide
VP-16	Etoposide

Abstract of Dissertation Presented to the Graduate School
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The prokaryotic type II DNA topoisomerase, DNA gyrase, is the target of the 4-quinolone antibiotics nalidixic acid and ciprofloxacin. These inhibitors have recently been shown to inhibit both mitochondrial DNA (mtDNA) synthesis in vitro and mammalian cell growth. The following studies present evidence that 4-quinolones cause a selective loss of mtDNA in vivo. Futhermore, drug induced loss of mtDNA appears to be mediated through the inhibition of a mitochondrial topoisomerase II-like enzyme.

Continuous exposure of mammalian cells to nalidixic acid or ciprofloxacin caused a selective loss of mtDNA from cells. Associated with this loss of mtDNA was a loss of mitochondrial function and cell viability. Other topoisomerase II inhibitors including dequalinium, ethidium bromide, Hoescht H33258, and pentamidine were also found to deplete cells of their mtDNA.

Previous studies have demonstrated that the 4-quinolones can inhibit eukaryotic nuclear DNA topoisomerase II in vitro. Therefore, it was important to distinguish between a nuclear- and/or mitochondrial-mediated mechanism of cytotoxicity. Ciprofloxacin did not induce cleavage of nuclear DNA, suggesting that nuclear topoisomerase II was not involved in the cytotoxic mechanism of this drug. Furthermore, VPM^{R}5 cells, a cell line resistant to the nuclear topoisomerase II anticancer drugs, were not resistant to ciprofloxacin, suggesting a different cytotoxic mechanism. Cells treated with ciprofloxacin were found to be resistant to the cytotoxic effects of this drug when grown under respiration-independent conditions, suggesting that inhibition of mitochondrial function was the cytotoxic lesion. These results rule out a nuclear mediated cytotoxic mechanism and support the hypothesis that ciprofloxacin induced cytotoxicity is the result of inhibition of mitochondrial function.

Analysis of mtDNA in drug treated cells demonstrated the existence of double-stranded site-specific mtDNA breaks, suggesting the possibility that a DNA topoisomerase II enzyme was the target of these drugs. These breaks in mtDNA were found have protein linked to the 5', but not the 3' ends. These results suggest that the depletion of mtDNA and loss in cell viability is due to the inhibition of a novel mitochondrial DNA topoisomerase II-like activity.

CHAPTER 1

INTRODUCTION

General Introduction

Mitochondria are organelles that are primarily responsible for the generation of energy in eukaryotic cells. They house the respiratory enzymes involved in energy production (oxidative phosphorylation) as well as a genome that encodes 13 of the proteins required for this process (Attardi and Shatz, 1988). The rest of the proteins involved in oxidative phosphorylation are encoded by the nuclear DNA and are imported into the mitochondria from the cytosol. Both nuclear and mitochondrial encoded proteins must be regulated in coordination to ensure equimolar complementation of the respiratory apparatus. Processes that modulate mitochondrial DNA (mtDNA) content and mitochondrial RNA (mtRNA) expression are critical to the understanding of mitochondria and their contribution to eukaryotic life. In addition to their role in energy production, mitochondria are responsible for many key reactions of intermediary metabolism including Beta-oxidation of fatty acids, heme biosynthesis, amino acid synthesis, and pyrimidine synthesis.

Identification of the regulatory processes, as well as drugs that interfere with these processes, should help unfold the mysteries of mitochondrial maintenance and propagation. To this end, these processes may be exploited for direct benefit to man. Drugs that target these processes may have beneficial anticancer effects. Several anticancer agents are thought to mediate their chemotherapeutic effect through a mitochondrial mechanism (Segal-Bendirdjian et al., 1988; Weiss et al., 1987; Chen and Cheng, 1989). These processes may also be unintentional targets of drugs, producing toxic side effects that limit the use of these potentially therapeutic agents (Arnaudo et al., 1991). Mechanistic studies involving drugs that affect mitochondrial regulatory processes may allow researchers to design safer, more effective pharmaceuticals.

Type II DNA topoisomerases are implicated in many nuclear processes including DNA synthesis, RNA transcription, and chromosomal organization (Noguchi et al., 1983; Nelson et al., 1986a and 1986b; Ryoji et al., 1984; Earnshaw and Heck, 1985; Gasser et al., 1986). These enzymes are necessary to relieve topological problems that arise during elongation and termination phases of mtDNA replication (Clayton, 1982; Castora et al., 1983; Berk and Clayton, 1976). Although type II DNA topoisomerases are likely to play a similar role in the regulation of mtDNA content and mitochondrial expression, experimental evidence is lacking. Understanding the function of these enzymes in the regulating mtDNA content and mitochondrial expression would be greatly facilitated by identification of inhibitors of a mitochondrial type II DNA topoisomerase.

This dissertation presents evidence demonstrating that the bacterial topoisomerase II inhibitors nalidixic acid and ciprofloxacin cause a selective loss of mtDNA from mammalian cells. Futhermore, this loss of mtDNA appears to be mediated through the inhibition of a mitochondrial DNA topoisomerase II-like enzyme.

Topoisomerases

Topoisomerases are enzymes that change the three-dimensional structure of DNA (reviewed in Liu, 1989). They perform this function through a DNA breakage-reunion reaction. Type I DNA topoisomerases catalyze a reaction that produces single stranded breaks in duplex DNA. Before rejoining these breaks, the enzyme passes the intact complimentary DNA strand through the breaks resulting in changes in the topological linking number (Wang, 1981). Type II DNA topoisomerases catalyze a reaction that produces a double stranded DNA break, through which another duplex DNA strand is passed before it is resealed (Liu et al., 1980). Type I topoisomerases change the topological linking number by intervals of one, while type II topoisomerases change the topological linking number by intervals of two (Wang, 1981; Liu et al., 1980; Hsieh and Brutlag, 1980; Miller et al., 1981).

It is clear that type II topoisomerases are important regulators of DNA metabolism (Wang, 1985). Their mechanism allows type II topoisomerases to catalyze supercoiling/relaxation of DNA (Gellert, 1976; Osheroff et al., 1983; Schomburg and Grosse, 1986), knotting/unknotting of DNA (Hsieh, 1983; Liu et al., 1980), and catenation/decatenation of circular DNA molecules (Hsieh and Brutlag, 1980; Miller et al., 1981). Hence, it is not surprising that topoisomerases are implicated in cellular processes such as DNA replication, transcription, segregation of daughter chromosomes during mitosis, and recombination of DNA (Noguchi et al., 1983; Nelson et al., 1986a and 1986b; Ryoji and Worcel, 1984; Earnshaw and Heck, 1985; Gasser et al., 1986). In fact, type II DNA topoisomerases are major components of the nuclear matrix suggesting a role for these enzymes in chromosomal organization (Earnshaw et al., 1985).

DNA gyrase (bacterial topoisomerase II) is unique in that it can introduce negative supercoils into DNA (Gellert et al., 1976; reviewed in Gellert, 1981). It fuels this reaction through hydrolysis of ATP. In the absence of ATP, it relaxes duplex DNA molecules. DNA gyrase is a tetramer composed of two alpha and two beta subunits. The A subunit (the gyrA gene product with a molecular mass of approximately 105 kilodaltons) contains the quinolone sensitive region of the enzyme and cleaves DNA (Cozarrelli, 1980). The B subunit (the gyrB gene product with a molecular mass of approximately 95 kilodaltons) contains the coumarin sensitive region of the enzyme and is responsible for hydrolysis of ATP (Cozzarelli, 1980).

Eukaryotic type II DNA topoisomerase has been highly conserved throughout evolution and is thought to have evolved from the fusion of bacterial genes, gyrA and gyrB, to form an enzyme consisting of two homologous 170 kilodalton subunits (Wyckoff et al., 1989). Eukaryotic type II topoisomerase is similar to DNA gyrase in that it can relax duplex DNA through an ATP-dependent, double-stranded DNA breakage-reunion reaction. Unlike DNA gyrase, eukaryotic type II topoisomerases cannot introduce supercoils into DNA (Wang, 1985).

The isolation and characterization of mitochondrial type II topoisomerases have been elusive. Several groups have attempted to purify a mitochondrial type II topoisomerase from mammalian cells but have been unsuccessful. However, a type II topoisomerase was purified to homogeneity from the kinetoplast of the trypanosomatid Crithidia fasiculata (Melendy and Ray, 1989). The kinetoplast is an organelle in trypanosomatids similar to mitochondria in mammalian cells. This enzyme was biochemically and physically distinct from the nuclear topoisomerase II in trypanosomatids (Melendy et al., 1988). Using a monoclonal antibody specific for the purified protein, immunohistochemical analysis identified the presence of this enzyme in the kinetoplast of the trypanosomatid but not in the nucleus. Furthermore, this enzyme was sensitive to the DNA gyrase inhibitor nalidixic acid at concentrations that affect bacterial growth, suggesting that a unique topoisomerase II-like activity exists in the kinetoplast of trypanosomes.

Other investigators have identified a type II topoisomerase activity in the trypanosomatid Tyrpanosoma equiperdum (Shapiro et al., 1989). Interestingly, the antitrypanosomal drug pentamidine induced double-stranded site-specific DNA strand breaks in kinetoplast DNA but not nuclear DNA. The 5' ends of these breaks were covalently linked to protein, suggesting that the kinetoplast type II topoisomerase activity is pharmacologically distinct from the nuclear type II topoisomerase. Also, this enzymatic activity was sensitive to the eukaryotic nuclear topoisomerase II anticancer drug, VM-26, suggesting that this enzyme has some similarities to the nuclear form.

The only studies on mammalian mitochondrial type II topoisomerase were performed by Castora et al. (1983). These investigators have identified an ATP dependent decatenating activity from rat liver mitochondria. In a partially purified enzyme preparation, this ATP-dependent relaxing activity was shown to be inhibited by the nuclear type II topoisomerase anticancer drugs, mAMSA and VM-26 (Lin and Castora, 1991).

Other topoisomerases have been identified in both prokaryotic as well as eukaryotic organisms. E. coli has been shown to contain topoisomerase related genes. These include parC and parE which have been shown to have amino acid sequence homology with the alpha and beta subunits of DNA gyrase, respectively (Kato et al., 1990). These new topoisomerases, termed type IV topoisomerases, have been shown to play an important role in chromosomal segregation (Kato et al., 1990; Kato et al., 1988). Saccharamyces cerevisiae has also been shown to contain a gene that is homologous to eubacterial DNA topoisomerase I and has been termed DNA topoisomerase III (Wallis et al., 1989). Also, the yeast gene, HPR1, may be yet another topoisomerase (Aguilera and Klein, 1990; Wang et al., 1990). Two forms of the eukaryotic topoisomerase II gene have been cloned from human cells, p170 (170 kilodaltons) and p180 (180kilodaltons). The p180 form of the enzyme has been shown to have both antigenic and fuctional differences from the p170 enzyme. Other forms of topoisomerases include reverse gyrase of S. acidocaldarius (which can introduce positive supercoils into DNA), topoisomerase II' from E. coli, and the Tn3 transposon-encoded topoisomerase (Kikuchi and Asai, 1984; Gellert et al., 1979; Krasnow and Cozzarelli, 1983). Identification of these topoisomerases suggest the possibility that different topoisomerases perform distinctly different cellular functions. Futhermore, these data suggest that mitochondria may contain a unique type II topoisomerase that is different from the nuclear form.

Catalytic Mechanism of Type II Topoisomerases

Type II DNA topoisomerases are enzymes that are responsible for changing the three dimensional structure of DNA. These enzymes perform this function through a DNA breakage-reunion reaction. This mechanism involves a covalent DNA-enzyme intermediate (Gellert, 1981; Wang, 1982; Vosberg, 1985) termed the "cleavable-complex" (Nelson et al., 1984) in which the enzyme attaches to the 5' ends of the cleaved DNA (Osheroff, 1987) through a phosphotyrosine linkage (Rowe et al., 1986).

Osheroff (1989) has divided the catalytic mechanism of these enzymes into five reactions including: 1) noncovalent binding, 2) DNA cleavage, 3) ATP binding and DNA strand passage, 4) religation, and 5) ATP hydrolysis. First, the enzyme becomes noncovalently associated with DNA (Osheroff, 1986; Sander et al., 1987). Then, in the presence of a divalent cation such as magnesium, double-stranded DNA cleavage occurs with covalent attachment of the topoisomerase subunits to the 5' ends of the DNA (Osheroff, 1987). ATP binding occurs next followed by the passage of a second strand of duplex DNA through the break site (Osheroff et al., 1983; Hsieh and Brutlag, 1980). Following strand passage, DNA strands are rejoined, and the enzyme remains noncovalently bound to DNA (Osheroff, 1986). After ATP hydrolysis, the enzyme dissociates from DNA and is ready for another round of catalysis (Miller et al., 1981; Osheroff et al., 1983). These enzymes are the target of several classes of anticancer agents that interfere with the religation step of catalysis resulting in the stabilization of the "cleavable-complex" (reviewed in Liu, 1989). These agents have become important therapeutic agents in the treatment of cancer.

Topoisomerases as Drug Targets

DNA topoisomerases are the cytotoxic target of several antibacterial and anticancer agents (Liu, 1989; Drlica and Franco, 1988). DNA topoisomerase II targeting anticancer agents inhibit topoisomerization by interfering with the religation step of catalysis, stabilizing the enzyme in a state called the "cleavable-complex" (Nelson et al., 1984). In this state, enzyme is covalently attached to the 5' ends of DNA. Stabilization of these "cleavable-complexes" is thought to be a neccessary step in the cytotoxic action of these agents (Glisson et al., 1986b; Sullivan et al., 1989; Ross, 1985; Davies et al., 1988). Eukaryotic type II topoisomerases are sensitive to a variety of compounds that have significant antitumor activity in humans (Liu, 1989). These drugs include the acridines (e.g., m-AMSA), anthracyclines (e.g., adriamycin), ellipticines, and the semisynthetic epipodophyllotoxins (e.g., VP-16 and VM-26) (Liu, 1989).

Evidence that cleavable complex formation is crucial in mediating the cytotoxic action of DNA topoisomerase II targeting anticancer drugs comes from studying drug-resistant mutant cell lines. VPMR5 cells, a CHO cell line that contains a mutation in the topoisomerase II gene, are no longer sensitive to the effects of several anticancer agents that target topoisomerase II, including m-AMSA, VP-16, adriamycin, daunomycin, and mitoxantrone. This mutant enzyme remains active in the presence of drug (Glisson et al., 1986b; Sullivan et al., 1989). To examine whether cleavable-complex formation or inhibition of topoisomerase II function was the dominant action involved in cytotoxicity, an experiment was performed with the fusion product of VPM^R5 cells and wild-type CHO cells. If inhibition of topoisomerase II was the sole effect that mediated cytotoxicity, then hydrid cells containing both mutant and wild-type topoisomerase II enzymes should become resistant to these drugs. Resistance should occur because the mutant enzyme in these hybrid cells would now perform the functions blocked by inhibition of the wild type enzyme by drug. However, these cells still died in the presence of these drugs, suggesting that formation of cleavable complexes was the dominant cytotoxic event rather than inhibition of the enzyme.

Cleavable complexes are rapidly reversed upon drug removal. The formation of drug induced DNA lesions are enough to generate a cytotoxic response (Osheroff, 1989a; Liu et al., 1983; Ross et al., 1979). Evidence also suggests that events secondary to cleavable-complex reversal are also important in cell death (Kupfer et al., 1987; Chow et al., 1988). Therefore, cleavable-complex formation is essential but not sufficient to produce a cytotoxic event.

Prokaryotic topoisomerases have become important antibacterial targets. Bacterial type II topoisomerase, DNA gyrase, is the target of quinolone antibacterial agents (Wang, 1985). Quinolones have become important clinical agents in the treatment of diseases caused by susceptable microorganisms (Zimmer et al., 1990; Andriole, 1988). Quinolones interact with the alpha subunit (gyrA gene product) of DNA gyrase which covalently attaches to the DNA during cleavable-complex formation (Sugino et al., 1978).

Effects of Bacterial DNA Topoisomerase II Inhibitors on Mammalian Cells

The 4-quinolones are antibiotics that target DNA gyrase, bacterial topoiosmerase II (Liu, 1989; Drlica and Franco, 1988). Quinolones have become important clinical drugs in the treatment of diseases caused by susceptable microorganisms. Their use in the clinic has increased significantly in the past decade. They are well tolerated and few adverse reactions are reported (Ball, 1986). Most adverse effects involve the gastro-intestinal system and include nausea, vomiting, diarrhea, and abdominal discomfort.

4-quinolones have been shown to inhibit mammalian cell growth in vivo and in vitro. In vivo, ciprofloxacin has been shown to inhibit bone marrow engraftment in irradiated mice that were chronically treated with high doses of ciproflxoacin (100 mg/kg/24 hrs) (Somekh et al., 1989a). The same group later reported that inhibition of bone marrow engraftment was short-lived and reversible (Somekh et al., 1989b).

Growth inhibitory effects of ciprofloxacin were also studied in vitro. Cell growth and immunoglobulin production in human lymphocytes were found to be inhibited by concentrations of ciprofloxacin over 40 μg/ml (Forsgren et al., 1987). It was demonstrated that ciprofloxacin stimulated thymidine uptake into human lymphocytes (Forsgren et al., 1987). If de novo pyrimidine biosynthesis was inhibited, cells would have to obtain pyrimidines from some other source. This finding by Forsgren et al. (1987) was consistent with ciprofloxacin inducing a block of de novo pyrimidine synthesis, stimulating thymidine uptake and incorporation into the cell. Further studies demonstrated that purine sythesis was unaffected (Forsgren et al., 1987). Dihydroorotate dehydrogenase is an enzyme involved in de novo pyrimidine biosynthesis. It is located in the inner mitochondrial membrane, and, in mammalian cells, is dependent on an active respiratory chain. Lyons and Christopherson (1990) demonstrated that ciprofloxacin did not affect dihydroorotate dehydrogenase directly during a 4 hour treatment at doses that inhibit mammalian cell growth, consistent with the findings of Forsgren et al (1987). Later, this same group reported that mitochondrial function was not inhibited by ciprofloxacin in studies utilizing isolated mitochondria (Riesbeck et al., 1990).

Mammalian cells can survive with a deficiency in pyrimidine nucleotide biosynthesis when the media is supplemented with uridine (Gregoire et al., 1984). If ciprofloxacin was inhibiting de novo pyrimidine synthesis, then cells grown in media containing uridine should be resistant to the growth inhibitory effects of this drug. Forsgren et al. (1987) tried unsuccessfully to overcome the growth inhibitory effects of ciprofloxacin by supplementing the media with uridine, suggesting that this was not the cause underlying the growth inhibitory effects of this drug.

In cell culture models, ofloxacin, perfloxacin, and nalidixic acid were shown to inhibit growth of myeloid precursor cells at concentrations over 70 μg/ml (Pessina et al., 1989). Another group reported a suppressive effect of 4-

quinolones on HeLa cell growth at concentrations over 100 times those required to kill bacterial cells (Oomori et al., 1988). Although no correlation between inhibition of mammalian cell growth and DNA gyrase inhibition was found, the authors did report that the growth inhibitory effects of 4-quinolones correlated with the concentrations of drug required to inhibit purified eukaryotic nuclear DNA topoisomerase II in vitro.

The 4-quinolones have been demonstrated to inhibit the catalytic activity of eukaryotic nuclear topoisomerase II in vitro. Studies by Bredberg et al. (1991) have suggested that growth inhibitory effects of ciprofloxacin may be due to formation of nuclear DNA strand breaks. They reported that ciprofloxacin at 80 µg/ml produced the same number of nuclear DNA strand breaks as 1.25 µg/ml VP-16, a nuclear DNA topoisomerase II poison. These authors suggested that nuclear DNA breaks were mediated by a topoisomerase II enzyme. However, the concentration of VP-16 used in their experiments will produce a log kill with a 20 hour pulse treatment. If ciprofloxacin produced as many topoisomerase II mediated strand breaks as VP-16, then ciprofloxacin should kill cells during a pulse exposure. The growth inhibitory effects induced by ciprofloxacin require continuous exposure for long periods of time, inconsistent with data indicating this drug targeted a nuclear topoisomerase II enzyme (Somekh et al., 1989b). The mechanism underlying the growth inhibitory effects of the 4-quinolones remains unclear.

Mitochondria

Mitochondria are double membraned organelles present in nearly all eukaryotic cells (Attardi and Shatz, 1988). They are responsible for producing energy (in the form of ATP) through oxidative phosphorylation. Mitochondria also have an important role in the biosynthesis of pyrimidines, amino acids, phospholipids, nucleotides, folate coenzymes, heme, and urea.

The inner mitochondrial membrane is an impermeable barrier between the mitochondrial matrix (inner fluid compartment) and the rest of the cell (Tzagoloff, 1982). Impermeability of inner mitochondrial membrane allows production of a proton gradient. Mitochondria use this gradient to drive protons back into the matrix by a carrier that converts ADP to ATP, thereby generating energy in the form of ATP (Mitchell, 1979; Boyer, 1987; Pedersen and Carafoli, 1987; Pedersen et al., 1987).

The evolution of bacteria to the mitochondrion has been explained by the embiosymbiotic theory (Gray, 1989). According to this theory, a primitive bacterium was engulfed by a protoeukaryotic cell. Through the course of time, the host cell became dependent upon the prokaryote for energy production. Conversely, the prokaryote lost many of its biosynthetic functions and became dependent upon the host in order to proliferate.

Many enzymes are involved in maintenance and function of mitochondria including enzymes involved in DNA replication, RNA transcription, and protein synthesis. Most of these enzymes are encoded in the nucleus and are imported into mitochondria from the cytosol. Many mitochondrial proteins and ribosomes have retained physical and biochemical similarities to their bacterial ancestors. Bacterial and mitochondrial ribosomes have striking genetic similarities. Similarities between bacteria and mitochondria are illustarted by the fact that many drugs that affect bacterial enzymes also affect their mitochondrial counterpart. Chloramphenicol and tetracycline, two inhibitors of bacterial protein synthesis also inhibit mitochondrial protein synthesis (Van Den Borgert and Kroon, 1981).

Physical Properties of Mitochondrial DNA

Mitochondria contain their own genome. The first discovery that mammalian mitochondria contained DNA came from Nass and Nass (1963). The mitochondrial genome has been completely sequenced from bovine, mouse, and human mitochondria (Bibb et al., 1981; Clayton, 1982; Parker and Watson, 1977). Mammalian mtDNA exists as a circular duplex DNA molecule of approximately 16 kilobases (Clayton, 1982). It encodes 13 proteins involved in oxidative phosphorylation including subunits 1, 2 and 3 of cytochrome c oxidase; apocytochrome b; subunits 1 through 6 of NAD dehydrogenase; and subunits 6 and 8 of H^+-ATPase (Figure 1-1) (Attardi and Shatz, 1988). Mammalian mtDNA also contains 14 tRNA genes and 2 rRNA subunits (12S and 16S). Another feature of mammalian mtDNA is that it exists at a large copy number (1000-2000 copies) inside the cell (Clayton, 1982; Bogenhagen and Clayton, 1974). Even though there are a large number of mitochondrial DNA molecules inside each cell, it still only accounts for less than one percent of the cell's total DNA. The mitochondrial genetic code is unique from the nuclear code (Lagerkvist, 1978).

The two strands of mtDNA can be separated from each other by differences in their bouyant densities, due to the proportion of purines in one strand compared to the other. These strands are named for the differences in their densities, the heavy strand (H-strand) and the light strand (L-strand) (Bogenhagan and Clayton, 1978a). The origin of replication within the H-strand contains a triplex DNA stucture called the D-loop. The D-loop is composed of approximately 1000 base pairs and serves as a primer for mtDNA replication.

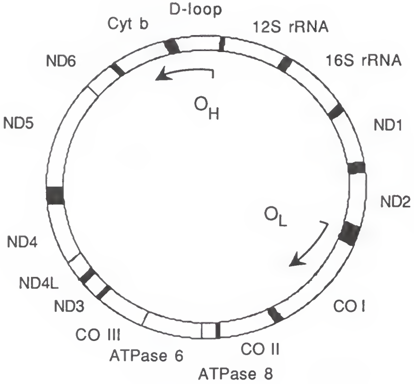


Figure 1-1 -Mammalian Mitochondrial DNA Map

Black squares represent tRNA genes. O_H: origin of heavy strand synthesis; O_L: origin of light strand synthesis; 12S rRNA, and 16S rRNA: 12S and 16S ribosomal RNA genes; CO I, CO II, and CO III are subunits I, II, and III of cytochrome c oxidase; ATPase 6 and ATPase 8: subunits 6 and 8 of H^+-ATPase; ND1, ND2, ND3, ND4, ND4L, ND5, and ND6: subunits of NADH dehydrogenase (modified from Attardi and Shatz, 1988).

Within the D-loop structure lies both heavy and light strand promoters for mitochondrial RNA synthesis (Chang and Clayton, 1984). From these promoters, transcription is thought to continue around the entire genome as a single polycistronic transcript. As the RNA is being transcribed, it is cleaved to make mature RNA species before the entire transcript is completed (Ojala et al., 1981).

Mitochondrial DNA is thought to be located in close association with the inner mitochondrial membrane. Albring et al. (1977) have shown that a protein associated with the membrane remains bound to mtDNA from HeLa cells lysed with Triton X-100 in the presence of 0.5 M salt. It was shown to bind to the mtDNA specifically at the origin of replication, suggesting a structural and/or regulatory role of this protein.

Replication of Mitochondrial DNA

Replication of mtDNA is a multi-step process including initiation, elongation, and termination (reviewed in Clayton, 1982). This mtDNA exists as a supercoiled DNA molecule containing approximately 100 negative superhelical turns (see figure 1-2). Initiation involves the synthesis of a 1000 base pair polynucleotide segment, resulting in the formation of a D-loop triplex structure, which serves as a primer for futher mtDNA synthesis (Ter Schegget et al., 1971; Eichler et al., 1977; Korn et al., 1978). Most mtDNA molecules (>75%) of mouse L-cells occur as D-loop forms. However, this D-loop structure is unstable with a half-life of approximately one hour. Then, the 1000 base pair primer is removed and a new primer synthesized (Bogenhagen and Clayton, 1978b).

After successful priming of the mtDNA, elongation begins (Figure 1-2). Replication of the heavy strand begins at the D-loop and continues unidirectionally for approximately 67% of the molecule after which the origin of light strand synthesis is exposed. This allows initiation of DNA synthesis upon the light strand in the opposite direction of the heavy strand (Martens and Clayton, 1979; Nass 1980a, 1980b; Bibb et al., 1981; Tapper and Clayton, 1981). Complete replication of the mtDNA requires 1 hour. Another hour is required to incorporate the 100 negative superhelical turns into the newly replicated daughter molecules (Bogenhagen and Clayton, 1978a).

Relatively little is known about the termination stage of mtDNA replication, but a type II topoisomerase is thought to be required (Bogenhagen and Clayton, 1978a). Replication of a circular molecule, like the mtDNA, results in formation of two interlocked daughter molecules (Clayton, 1982; Berk and Clayton, 1976). Interlocked molecules must be separated in order to pass one of the newly replicated DNA molecules to each of the daughter cells during cell division. In order to resolve these catenated rings of DNA, it is necessary to break the phosphodiester backbone of one of the molecules, pass the second molecule's DNA through this break site, and then reseal the break site. Thus, due to the circular nature of mtDNA, it is thought that a topoisomerase II activity must exist in mitochondria. Whether or not mitochondrial DNA topoisomerase II is different from nuclear DNA topoisomerase II has been a subject of much debate. As stated above, a mitochondrial type II DNA topoisomerases has been identified in trypanosomatids (Melendy et al., 1988; Shapiro et al., 1989). However, investigators have been unsuccessful in isolating a mitochondrial type II topoisomerase in mammalian cells (Castora et al., 1983; Lin and Castora, 1991).

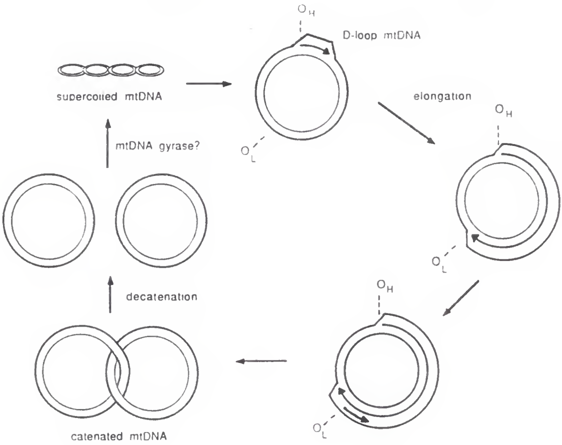


Figure 1-2 -Replication of Mammalian Mitochondrial DNA

O_H: origin of heavy strand synthesis; O_L: origin of light strand synthesis.

Regulation of Mitochondrial DNA Content

Mitochondrial DNA expression is regulated differently from nuclear DNA expression. In order to increase production of mitochondrial proteins, the cell must increase mitochondrial RNA production. Cells can increase mtRNA production in two ways. They can either increase the rate of transcription of the mitochondrial genome or increase the copies of mtDNA molecules avaliable for transcription. It has been shown that mtDNA genome multiplication is the main regulatory event in modulating mitochondrial expression, suggesting that the only way the cell can increase transcription of the mitochondrial genome is to increase mtDNA copy number (Williams, 1986; Williams et al., 1986). Conditions that modulate mtDNA content may have dramatic consequences on cellular metabolism and possibly restrict the ability of cells to adapt to new environments. Therefore, regulation of the mtDNA copy number is critical in maintaining adequate expression of the mitochodnrial genome.

One way the cell can increase its oxidative capacity is to amplify the mtDNA copy number, thereby increasing the gene dosage. This was demonstrated by measuring the mtDNA copy number and expression of mitochondrial rRNA and mRNA synthesis in striated skeletal muscle subject to prolonged electrical stimulation (Williams, 1986). In these experiments, the author reported an average 1.5- to 2-fold increase in both mtDNA copy number and mitochondrial rRNA/mRNA expression in muscle cells subject to continuous electrical stimulation for 10 days. Individual samples were shown to increase the mtDNA copy number and mtRNA expression up to 8-fold. In another set of experiments, Williams et al. (1986) demonstrated that expression of enzymes involved in glycolysis were repressed during a 21 day electrical stimulation regimen. Again, mtDNA copy number increased 4-fold over nonstimulated muscle. Neither the mtDNA copy number nor mtRNA expression changed with a 5 day regimen, suggesting that compensatory effects of mtDNA amplification are only involved in long term regulation. Cells placed in hypoxic environments did not display a change in mtDNA copy number (Murphy et al., 1984). However, these cells were only placed in hypoxic conditions for 4 days. Like that observed in muscle contraction experiments described above, regulation of mtDNA copy number may require long-term environmental exposures.

One group has shown that a cytoplasmic hybrid cell line can modulate its mitochondrial population depending upon environmental selection with rutamycin, a mitochondrial ATPase inhibitor (Tonsgard et al., 1990). Researchers fused a nucleated cell from a rutamycin sensitive cell line with a cytoplasm from a rutamycin resistant cell line. These hybrid cells contained both sensitive and resistant types of mitochondria. Moreover, rutamycin resistance has been shown to be cytoplasmically inherited (Lichtor et al., 1979). After these cytoplasmic hybrids were passaged in the absence of rutamycin for extended periods of time, they still retained the ability to grow in the presence of rutamycin (Tonsgard et al., 1990). However, cell growth was delayed approximately two days. These investigators suggested that even though the resistant population of mitochondria was initially low, it expanded during the two days when growth was inhibited. After the resistant population of mitochondria reached a sufficient level, cell growth resumed. These data suggest that cells can adapt by expanding different mitochondrial populations in response to environmental pressures.

Mitochondrial DNA copy number has been shown to vary during differentiation and de-differentiation (Singh and Veltri, 1991). In fact, different mammalian organs such as heart, liver, kidney, and brain have been shown to contain a distinct mtDNA copy number (Veltri et al., 1990). Aquisition of anchorage-independent growth is one characteristic during the transformation process in carcinogenesis. During this process, cells change to a less differentiated type to allow cells to divide. Interestingly, one study exploring the role of mtDNA on growth phenotypes demonstrated that mtDNA and transformation were somehow linked (Zinkewich-Peotti et al., 1990). In these studies, the authors found a correlation of the loss of anchorage-independent growth and mtDNA depletion in immortal avian cells, suggesting the presence of mtDNA may be required to maintain the transformed phenotype of avian cells. Therefore, the development of drugs that target mtDNA may become useful chemotherapeutic agents.

Mitochondrial DNA content can also be modulated through use of xenobiotics. Several agents have been shown to deplete cells of their mtDNA (Weisman and Attardi, 1987; Nass, 1984; Arnaudo et al, 1991; Segal-Bendirdjian et al., 1988). These include ethidium bromide (an intercalator), MGBG (a polyamine analogue), AZT (a nucleoside analogue with significant anti-HIV activity), and ditercalinium (a bisintercalator). Many of these compounds are thought to deplete cells of their mtDNA by inhibiting mtDNA synthesis. Nuclear DNA synthesis is not significantly affected, thereby causing a dilution of mtDNA through each successive cellular division. These findings suggest that mitochondrial DNA synthesis has specific regulatory processes distinct from the nuclear processes that can be selectively inhibited by drugs.

Aims of this Study

Investigation of the cellular processes that regulate mtDNA copy number will facilitate an understanding of the events involved in mitochondrial biogenesis. More importantly, studying agents that impact on the regulation of mtDNA content will help dissect processes involved in mtDNA metabolism. DNA topoisomerase II is a likely player in mtDNA metabolism (Wang, 1985). It is involved in nearly all stages of nuclear DNA metabolism. Mitochondrial DNA is an interesting molecule to study from a topological point of view. This genome has no free ends and, therefore, the only way to change the linking number of this molecule is to produce transient DNA strand breaks, because there are no DNA ends to rotate freely. Clearly, removal of the supercoils generated by DNA replication and transcription must be performed or else torsional stress would limit the movement of replication and transcription complexes along the DNA (Wu et al., 1988). DNA topoisomerase II becomes even more interesting due to the fact that several classes of antibacterial and anticancer agents have been shown to target type II DNA topoisomerases in prokaryotes and eukaryotes, respectively. The possibility of a distinct mitochondrial type II DNA topoisomerase comes from the fact that the bacterial DNA topoisomerase II and eukaryotic nuclear DNA topoisomerase II are pharamacologically distinct from one another (Huff and Kreuzer, 1990). The eukaryotic mitochondrial topoisomerase may have bacterial origins that are suggested by the embiosymbiotic theory of mitochondrial evolution (Gray, 1989).

At the onset of this study, little was known about mammalian mitochondrial type II topoisomerases. Only a description of an ATP-dependent decatenating activity in liver mitochondria was presented (Castora et al., 1983). Since then, a type II DNA topoisomerase was isolated from mitochondria in trypanosomatids (Melendy et al., 1988; Shapiro et al., 1989). Therefore, our goal was to directly demonstrate existence of a type II topoisomerase enzyme in mammalian mitochondria. To establish this, an inhibitor was sought (and found) to help covalently trap such an enzyme directly upon mtDNA itself. Also, the cellular consequences of these inhibitors were investigated. Theoretically, an inhibitor of a mitochondrial type II DNA topoisomerase should have an impact on mtDNA content. Disruption of the equilibrium of mtDNA content in mammalian cells should have dramatic consequences on cell viability.

Specifically, our aims were as follows:

1. To determine if chronic exposure to nalidixic acid and ciprofloxacin affect mammalian mtDNA content.
2. To assess potential cytotoxic consequences of mtDNA depletion and establish a causal relationship between mtDNA depletion and loss of cell viability.
3. To demonstrate directly the existence of a type II DNA topoisomerase located in mitochondria.
4. To determine whether or not this mitochondrial topoisomerase II enzyme is sensitive to nalidixic acid and ciprofloxacin thereby identifying a possible molecular mechanism by which mtDNA depletion occurs.

CHAPTER 2

MATERIALS AND METHODS

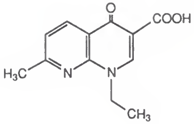
Materials

All cell culture reagents were obtained from GIBCO (Gaithersburg, MD). All other reagents and chemicals were obtained from Sigma of Fisher and were the finest quality available.

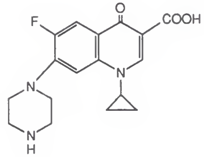
Drugs

Adriamycin, daunomycin, dequalinium, ethidium bromide, Hoescht H33258, nalidixic acid, and pentamidine were purchased from Sigma Chemical Company (St. Louis, MO). VM-26 and VP-16 were generously provided by Bristol-Meyers Co. (Syracuse, NY). m-AMSA, and o-AMSA were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Ciprofloxacin was a generous gift by Miles, Inc. (West Haven ,CT). Propidium iodide (Calbiochem; La Jolla, CA) was a generous gift from Dr. Allen Neims, Department of Pharmacology and Therapeutics, University of Florida. The chemical structures of these drugs are listed in figures 2-1, 2-2, and 2-3.

Nalidixic Acid



Ciprofloxacin



Epipodophyllotoxin

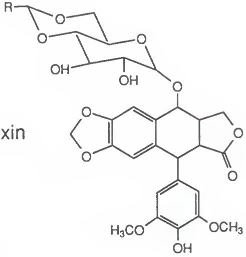
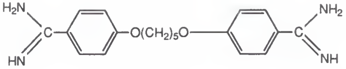
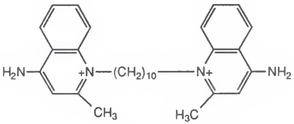


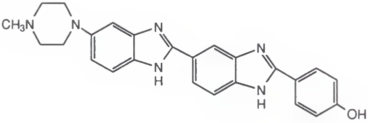
Figure 2-1 -Chemical Structures of Nalidixic Acid, Ciprofloxacin, and Epipodophyllotoxin



Pentamidine

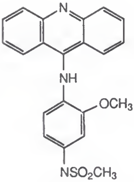


Dequalinium

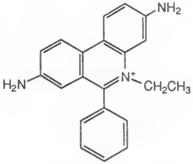


Hoechst H33258

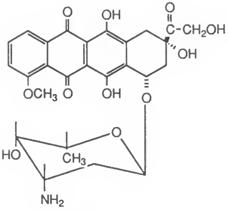
Figure 2-2 -Chemical Structures of Pentamidine, Dequalinium, and Hoescht H33258



m-AMSA



Ethidum Bromide



Adriamycin

Figure 2-3 -Chemical Structures of m-AMSA, Adriamycin, and Ethidium Bromide

Enzymes

Proteinase K, RNase T1, and lambda exonuclease was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Exonuclease III and Sac I restriction enzyme were purchased from Promega (Madison, WI).

Cell Culture

L1210 cells are a mouse leukemia cell line having a doubling time of 10 hr and were obtained from Dr. William Hauswirth, Department of Immunology and Medical Microbiology, University of Florida. L1210 cells were maintained as a suspension in minimal essential media (alpha modification from GIBCO) with either 10% fetal bovine serum or 10% horse serum (GIBCO) at 37°C under a humidified atmosphere containing 5% CO_2. Cells were counted with a Coulter Counter model Zf particle counter and maintained in logarithmic growth by reseeding at a density of 0.4 to 1 X 10^5 cells/ml.

B16 cells are a mouse melanoma cell line having a doubling time of 14 hr and were obtained from Dr. Tien-Wen Wiedmann, Division of Nuclear Medicine, Stanford University. HeLa S3 cells are a human cervical carcinoma cell line having a doubling time of 24 hr and were obtained from Dr. Bert Flanagan, Department of Immunology and Medical Microbiology, University of Florida. B16 and HeLa cells were maintained as sub confluent monolayer cultures in minimal essential media (alpha modification) with 10% fetal bovine serum at 37°C under a humidified atmosphere containing 5% CO_2. Cells were maintained in logarithmic growth by subculturing every other day. Cells were detached using Puck's A saline (5 mM KCl, 138 mM NaCl, 4 mM $NaHCO_3$ pH

7.4, 5.6 mM glucose) supplemented with 0.02% (w/v) EDTA and reseeded at a density of 5 X 10^5 in a 75 cm^2 tissue culture flask. In some experiments, HeLa cells were maintained in the absence of glucose as described by Wice et al. (1981). Glucose-free media (DMEM NG) obtained from GIBCO contained Delbecco's modified Eagle Media (DMEM) media lacking glucose, glutamine, phenol red, and sodium bicarbonate and was supplemented with 3.7 g/l sodium bicarbonate, 4 mM glutamine, 1 mM pyruvate, 6 g/l HEPES, 10 mM uridine, and 2% Ultraser G serum substitute (GIBCO). In these cases, the HeLa cells were subcultured using a trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA) from GIBCO.

CHO cells are a Chinese hamster ovary cell line having a doubling time of 24 hr and were obtained from Dr. Warren Ross, Department of Pharmacology and Therapeutics, University of Florida (Gupta, 1983). VPM^R5 cells are a CHO subline that are resistant to nuclear topoisomerase II drugs (see Gupta, 1983). CHO cells were maintained in DMEM supplemented with 10% fetal bovine serum, two times the normal GIBCO MEM amino acids, two times the normal GIBCO MEM vitamins, 6 g/l HEPES, 3.7 g/l sodium bicarbonate, and 40 mg/l L-proline at 37°C under a humidified atmosphere containing 5% CO_2. DMEM PUT media contained DMEM supplemented with 1 mM sodium pyruvate (GIBCO), 10 µg/ml uridine (Sigma), and 10 µg/ml thymidine (Sigma). CHO and VPM^R5 cellsdetached with Puck's A saline containing 0.02% EDTA to detach the cells from the flask and reseeding them at a density of 5 X 10^5 in a 75 cm^2 tissue culture flask containing fresh media.

Table 2-1 -Characteristics of Growth Medium for Mammalian Cells

	DMEM[d]	DMEM PUT[e]	DMEM NG[f]	α-MEM[d]
Glucose (g/l)	4.5	4.5	none	1
Pyruvate (mM)	none	1	1	1
Glutamine (mM)	1	1	4	1
Thymidine (mM)	none	0.04	none	none
Uridine (mM)	none	0.04	10	none
FCS	10%	10%	none	10%
Ultraser G[a]	-	-	+	-
Phenol Red	+	+	-	+
Extra (2x)				
Amino Acids[b]	+	+	-	-
Vitamins[c]	+	+	-	-

[a]-serum substitute from GIBCO catalog # 680-1810AE

[b]-MEM amino acids from GIBCO catalog # 320-1130AH

[c]-MEM vitamin solution from GIBCO catalog # 320-1120AG

[d]- requires active mitochondria for growth

[e]- supports respiratory independent growth

[f]- energy metabolism only through respiratory chain

All media was obtained from GIBCO:

DMEM (standard DMEM and DMEM PUT) media catalog # 430-2100EL

Standard DMEM media is defined as DMEM (high glucose) base supplemented with 40 mg/l L-proline, 6 g/l HEPES, 3.7 g/l sodium bicarbonate, 10% FCS, 2 times the normal MEM amino acids, and 2 times the normal MEM vitamins.

DMEM for DMEM NG (No Glucose) media catalog # 430-3800EB

α-MEM media catalog # 410-2000EL.

DNA Clones

The Alu nuclear repetitive DNA clone BLUR8 (Deininger et al., 1981) was obtained from Dr. L. Liu (Johns Hopkins University). The mouse Per nuclear repetitive DNA clone M2.5 R1 (Shin et al., 1985) was obtained from Dr. T. Bargiello (Rockefeller University). Figure 2-4 displays the genetic map of the mammalian mtDNA genome. The entire mouse mitochondrial genome, inserted into the Sac 1 site of pSP64, was obtained from Dr. W. Hauswirth at the University of Florida (Hauswirth et al., 1987). The mouse mitochondrial subclone pMBS2.1 contains the 2.1 kb Bam H1-Sac 1 fragment of the mouse mitochondrial DNA (figure 2-4) (Bibb et al., 1981) inserted into the Bam H1-Sac 1 site of pSP64. The human mitochondrial DNA clone pKB mbo I 2.8 was obtained from Dr. D. Clayton at Stanford University (figure 2-4). The clones were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mMole, New England Nuclear) using a nick translation kit obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Clonogenic Cytotoxicity Experiments

To assess the cytotoxic effects of drugs, either trypan blue exclusion or clonogenic cytotoxicity experiments were performed. Viable cells normally exclude trypan blue by an active efflux transport mechansim. When cells are no longer viable, they lose the ability to transport trypan blue out of the cell. Therefore, any cells that do not exclude trypan blue are not viable (Roper and Drewinko, 1976). To determine what fraction of a cellular population was viable, an 10 µl of cell culture media was diluted with 10 µl PBS (phosphate

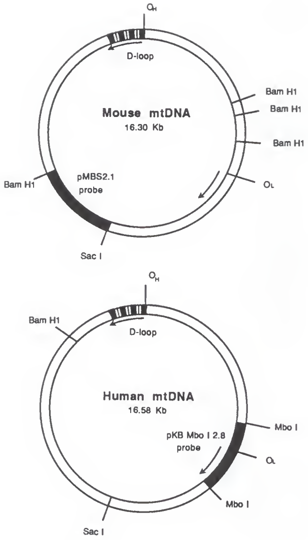


Figure 2-4 -Mitochondrial DNA Probe Maps

O_H- origin of heavy strand replication; O_L- origin of light strand replication; hatched region denotes the D-loop; solid region denotes the sequence of the mtDNA contained in the probes. Arrows show direction of replication from heavy or light strand origins.

Top map: Mouse mtDNA subclone pMBS2.1 contains the 2.1 kb Bam H1-Sac I fragment.

Bottom Map: Human mtDNA subclone pKB Mbo I 2.8 contains the 2.8 kb Mbo I fragment between Mbo I sites 10 and 11.

buffered saline: 10 mM NaHPO4, pH 7.4, 137 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4) containing 0.4% (w/v) trypan blue trypan blue, loaded onto a hemacytometer, and counted. A ratio of nonviable cells versus total cells counted was calculated and multiplied by 100 to yield a percentage.

In experiments to assess the antiproliferative effects of drugs, clonogenic cytotoxicity experiments were utilized. Cells were counted by hemacytometer and diluted to 300 cells/ml in media. One ml aliquots were pipetted into each well of a 6-well tissue culture plate. Cells were then incubated overnight at 37°C to allow attachment after which the media was removed by vacuum aspiration and replaced with 3 ml fresh media containing the appropriate concentration of drug. In experiments where cells were allowed to recover in drug-free media, the drug-containing media was removed and the cells were washed three times with 1 ml of 37°C PBS and then placed in 3 ml fresh drug-free media. After allowing the viable cells to grow into colonies (6-10 days), the plates were stained with crystal violet (2% (w/v) in methanol) and the colonies counted. In experiments in which B16 cells were used, the cells were allowed to grow for 6 days after drug treatment. In experiments in which HeLa or CHO cells were used, the cells were allowed to grow for 10 days after drug treatment.

Cell Fixing and Staining for FACS Analysis

Mouse L1210 cells (1×10^6) were pelleted at 1000 rpm for 2 min and washed in 1 ml PBS before resuspending in 500 µl of PBS. Cells were then incubated on ice for 15 min Ice cold 100% ethanol (500 µl) was then added and the ethanol mixed by pipetting air through the sample to avoid cell lysis. Samples were then incubated on ice for 15 min Samples were pelleted at 500 rpm (savant microfuge; HSR-12 rotor) for 3 min at room temperature. Pellets were washed once with 1 ml PBS before resuspending the samples in 125µl of RNase buffer (500 units/ml RNase T1, 1.12% sodium citrate) and incubated at 37°C for 10 min Cells were then stained by the addition of 125 µl propidium iodide (50 µg/ml in 1.12% sodium citrate) for 30 min at room temperature. Samples were then pushed through a 44 µm mesh nylon filter before FACS analysis by the University of Florida Flow Cytometry Core Facility using the SFIT modeling program.

Mitochondrial DNA Cleavage Assay

To detect cleavage of mtDNA, isolated mitochondria or whole cells were treated with 1% SDS, 20 mM EDTA pH 8 (final concentrations). Samples were then digested with 2 mg/ml proteinase K, 6 units RNase T1, and 5 mM DTT. After digestion at 37°C for overnight, DNA was purified by phenol, phenol/chloroform, and chloroform extractions and ethanol precipitation for restriction digestion followed by analysis by agarose gel electrophoresis and Southern blotting.

Mitochondrial Isolations

Mitochondria were isolated by differential centrifugation essentially as described by Hauswirth et al. (1987). Briefly, L1210 cells (1-2 X 10^7) were pelleted at 1000 x g and washed in TD buffer (134 mM NaCl, 2.5 mM Tris-HCl pH 7.5, 5 mM KCl, 0.7 mM Na_2HPO_4) before resuspending in 1 ml swelling buffer (10 mM NaCl, 10 mM Tris-Cl pH 7.5, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM PMSF, 0.1 mM EGTA, 1 mM EDTA) and allowed to incubate for 15 min on ice. Cells were then disrupted by dounce homogenization with 50 strokes after which KCl was added to a final concentration of 0.15 M. Nuclei were sedimented with a low speed spin (1000 x g) for 10 min at 4°C and the mitochondria in the resulting supernatant were placed into another tube and pelleted at 10,000 x g at 4°C for 15 min The mitochondrial pellet was then resuspended in 700 µl reaction buffer (10 mM NaCl, 10 mM Tris-Cl pH 7.5, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM PMSF, 0.1 mM EGTA, 0.1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 0.25 M sucrose). Aliquots of 30 µl of the mitochondrial suspension were typically used in each sample.

Isolation of Cellular DNA

In general, cells were collected by centrifugation at 1000 x g for 2 minutes at room temperature. Supernatants were discarded and the cell pellets resuspended in 400 µl of lysis buffer (20 mM Tris-Cl pH8, 20 mM EDTA, 1% SDS, and 400 µg/ml proteinase K) and incubated overnight at 37°C. Lysates were then phenol and ether extracted. Residual ether was removed under vacuum in a Savant speed-vac centrifuge and samples were digested with RNase T1 (6 units) for 2 hours at 37°C. Samples were again phenol and ether extracted, the DNA precipitated with 2.5 volumes of ethanol, and the resulting pellet resuspended in 200 µl of TE (10 mM Tris-Cl, pH8, 1 mM EDTA). The concentration of DNA was then determined spectrofluorometrically (Lebarca and Paigen, 1980) and equal amounts of DNA (10 µg) from each sample were analyzed by Southern blotting (see below).

Isolation of Protein-Linked DNA (Cleavable-Complexes)

Protein linked DNA was purified essentially as described by Shin and Snapka (1990). Cells to be filtered through glass fiber filters were labeled with 0.2 µCi/ ml ^{3}H-thymidine. Cell pellets were then lysed in 200 µl of 1% SDS, 20 mM Tris-Cl pH 8.0, 25 mM EDTA, 1 mM PMSF and either counted directly or filtered through GF/C glass filters (Whatman; Hillsboro, OR) for scintillation counting. Samples that were filtered were first diluted with 5 ml (plastic pipette) of 37°C protein binding buffer (PBB) which contained 0.4 M guanidine HCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.01% Sarkosyl, 0.3 M NaCl, 1 mM PMSF, and 1 mM DTT. Filters were then washed two times with 3 ml of PBB at 37°C before they were dried and placed in a scintillation vial with 5 ml scintiverse II scintillation fluid (Fisher Scientific; Fair Lawn, NJ).

Cell lysates were bound to glass beads (100 µl of 50% (w/v) suspension) in 1 ml PBB and incubated for 15 min at room temperature. Glass beads were pelleted at 5000 rpm (savant microfuge) for 2 min and the supernate discarded. Glass pellets were washed two times with PBB 15 min at 37°C with occasional mixing. Protein binding buffer was washed free from the glass with 1 ml of 3M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT which was mixed and pelleted without a 37°C incubation. Glass beads were then treated with proteinase K in 100 µl of elution buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 100 mM NaCl, and 0.1% SDS) and incubated at 37°C overnight. DNA was then eluted after a 15 min incubation at 65°C before pelleting the glass beads. Supernatants were removed and placed into another tube. Glass pellets were then re-extracted with 100 µl elution buffer at 65°C as above and the supernatants combined. In some cases, Glass pellets were resuspended in

10μl of elution buffer and proteinased overnight at 37°C. In these cases, one tenth of a volume of 10x loading buffer (50% sucrose, 0.05% bromophenol blue, 10 mM EDTA) was added to each sample and the complete sample (glass and eluate) was loaded directly into a well of an agarose gel and electrophoresed as described below.

Exonuclease Treatments

DNA eluted off glass beads were analyzed for orientation of the protein linkage. The linkage of the protein on the ends of the DNA was assessed with an exonuclease protection assay. DNA eluted off glass beads were prepared by two successive phenol extractions followed by a chloroform extraction. Yeast tRNA was included as a carrier at 10 μg/ml and the DNA precipitated by adding ammonium acetate to 0.25 M and 2.5 volumes of cold 95% ethanol. Samples were mixed well and placed at -80°C for 15 minutes and DNA pelleted at 15,000 rpm at 4°C for 15 minutes. Pellets were then washed two times with 200 μl ice cold 70% ethanol and dried under vacuum. Samples were then resuspended in 100 μl H_2O and 30 μl aliquots placed into 1.5 ml eppindorf tubes and 3.5 μl of 10x Exonuclease III buffer (50 mM $MgCl_2$, 10 mM DTT, and 500 mM Tris-Cl pH 8.0 final concentrations) or lambda exonuclease buffer (25 mM $MgCl_2$, 500 μg/ml BSA, 670 mM K-glycine pH 9.4 final concentrations) was added. Nuclease (1.5 units lambda exonuclease or 15 units exonuclease III) was added to samples and incubated at 37°C for 30 min before reactions were quenched with addition of one tenth of a volume of 250 mM EDTA, 1 % SDS before adding 1 unit of RNase T1. Samples were then incubated for an hour at 37°C. Loading buffer was added (0.1 volumes of a 10x stock) and samples were loaded onto a 0.7% agarose gel and electrophoresed described below.

Gel electrophoresis, Southern Blotting, and Autoradiography

Samples subject to standard horizontal gel electrophoresis were combined with 0.1 volumes of 10x loading buffer (50% sucrose, 0.05% bromophenol blue, 10 mM EDTA) and loaded onto a 0.7% agarose horizontal gel in TBE (90 mM Tris-borate, pH 8.3, 2 mM EDTA) (Manniatis et al., 1982). Following electrophoresis gels were stained with ethidium bromide and the DNA visualized using a UV transilluminator. DNA was transferred onto a nitrocellulose filter and hybridized to nick translated [^{32}P]-labeled mitochondrial-specific DNA probes as described by Sambrook et al. (1989). Radioactive mitochondrial signal was directly quantified from nitrocellulose filters using a Betascope 603 Blot Analyzer (Betagen Corporation) and then the filter was autoradiographed at -70°C using Kodak XAR 5 film and a DuPont Lightning-plus intensifying screen. Variations in total amount of DNA in each sample were corrected for by removing the mitochondrial DNA probe and rehybridizing the blot to nuclear-specific Per or alu DNA probes. Relative amount of total cellular DNA in each sample was also independently determined from desitometric tracings of photographic negatives of the ethidium bromide stained gels.

Cytochrome C Oxidase Assay

To assess mitochondrial function, cytochrome c oxidase (COX) activity was measured. Cytochrome c oxidase activity was determined using a spectrophotometeric assay (Segal-Berdirdjian et al., 1988). Cells (1 X 10^6) were harvested by centrifugation at 1000 x g and the supernatant discarded. Pellets were washed twice with 0.25 M sucrose before resuspending in 1 ml of

H_2O. Cells were then disrupted by 20 strokes in a tight fitting dounce homogenizer. To initiate to assay, 200 µl of the resulting homogenate was combine with 100 µl of 0.1 M potassium phosphate, pH 7, 70 µl of 1% reduced ferricytochrome c (Sigma type III), and 630 µl of H2O. Ferricytochrome c was reduced with 16 mM sodium ascorbate and purified over a Biogel P4 column. Reactions were incubated at 37°C and the absorbances were measured at 550nm over a 30 min period against a blank containing 1 ml of 0.01 M potassium phosphate pH 7, 0.07 % oxidized ferrocytochrome c (ferricytochrome c was oxidized by including 1 mM potassium ferricyanide in the blank). Cytochrome c oxidase activity was defined as the change in absorbance at 550 nm after a 30 min incubation and was expressed relative to untreated control cells.

Drug Uptake Experiments

To access the cellular levels of ciprofloxacin, a fluorometric assay was utilized based on the fact that ciprofloxacin is intrinsically fluorescent (Chapman and Georgopapadakou, 1989). The excitation maxima of ciprofloxacin is 335 nm and its emission maxima is 415 nm. CHO cells (6×10^7) detached with Puck's A saline containing 0.02% EDTA were pelleted at 1000 x g and the supernatant discarded. Cells were then resuspended in 6.5 ml of appropriate media and six 1 ml aliquots were placed into 15 ml tissue culture tubes. To each tube, 9 ml of fresh media containing various drug concentrations was added (25 µl of 40 mg/ml stock) were appropriate. Samples were then incubated in a 37°C water bath for 1 hr before placing samples in an ice slush in the cold room for 15 minutes. Samples were pelleted at 1000 x g for 5 min at 4°C and supernatants discarded. Resulting pellets were washed with ice cold

PBS (10 ml) two times. Final pellets were then resuspended in 2 ml of PBS and cell density counted by hemacytometer. Digitonin was added to 100µM (6.2 µl of a 4% w/v stock) and, after the samples were incubated at 20°C for 20 min, samples were centrifuged at 2500 rpm for 10 min Supernates were then analyzed by fluorescence spectroscopy. Samples were compared to a ciprofloxacin stock of known concentration and non-specific fluorescence was subtracted out using supernants from untreated cells.

Statistical Analysis

Statistical analysis was performed using a one-way ANOVA with student-Newman-Kuel's post-hoc test. Differences with p values below 0.05 were considered significant.

CHAPTER 3

EFFECTS OF CIPROFLOXACIN ON MITOCHONDRIAL DNA CONTENT

Introduction

The 4-quinolones are a class of antibacterial drugs that act by inhibiting the type II topoisomerase, DNA gyrase (Drlica and Franco, 1988; Gootz et al., 1990). At very high concentrations (approximately 100-fold higher that those required to inhibit bacterial growth), these agents inhibit growth of mammalian cells (Hussy et al., 1986). There is some evidence that inhibition of mammalian cell growth may be related to the effects these drugs have on mitochondrial DNA (mtDNA) synthesis. Castora et al. (1983) have shown that replication of mtDNA in isolated rat mitochondria is inhibited in the presence of the 4-quinolone drugs, nalidixic acid and oxolinic acid. They suggested that these drugs might be inhibiting a mitochondrial DNA gyrase-like enzyme that functions to facilitate changes in the topology of mtDNA that are required for replication. This is consistent with the studies in the trypanosomatid Crithidia fasciculata that have identified a mitochondrial type II topoisomerase that is inhibited by the 4-quinolone, nalidixic acid (Melendy and Ray, 1989). Other groups have reported that nalidixic acid inhibited mtDNA synthesis in yeast cells and isolated mammalian mitochondria (Luha et al., 1971, Castora et al., 1983). These studies led us to investigate the relationship between inhibition of mammalian cell growth and alterations in mtDNA content using mouse leukemia L1210 cells as a model.

Results

To determine if nalidixic acid affected mtDNA content in mammalian cells, mouse L1210 cells were grown in the continuous presence of nalidixic acid (250 μg/ml) for 14 days. At each time point, the cell number was assessed by Coulter counter, and an aliquot of cells harvested to assess the mtDNA content. Another aliquot was reseeded into a new flask containing fresh media with the appropriate concentration of drug. As can be seen in Figure 3-1, cell growth in the presence of nalidixic acid continued unchanged for two days. After two days, growth of nalidixic acid treated cells slowed down. The inhibitory effect of nalidixic acid on cell growth was found to be reversible when cells treated for six days with drug were placed in drug-free media. Full recovery took between 4 and 7 days. The effect on cell growth was dose dependent with an IC_{50} value (concentration that inhibits cell growth by 50%) of 250 μg/ml (data not shown).

To determine if nalidixic acid had an effect on mtDNA content during continuous drug exposure, total cellular DNA was isolated from mouse L1210 cells treated with nalidixic acid (250 μg/ml) for various periods of time. Equal amounts of DNA were loaded onto an agarose gel and electrophoresed, blotted to nitrocellulose, and hybridized to labeled mouse mtDNA. After mtDNA quantification, a second hybridization to a nuclear DNA probe was performed to correct any differences in total DNA content in each lane. After one day of drug exposure, a 60% reduction in the mtDNA level was observed (Fig. 3-2A, lane 2; Fig. 3-2B). The mtDNA specific signal did not decrease below 20% and stayed at 20% for the remainder of the experiment (Fig. 3-2A lanes 3-7). If cells were placed in fresh drug-free media after six days of drug exposure, mtDNA content

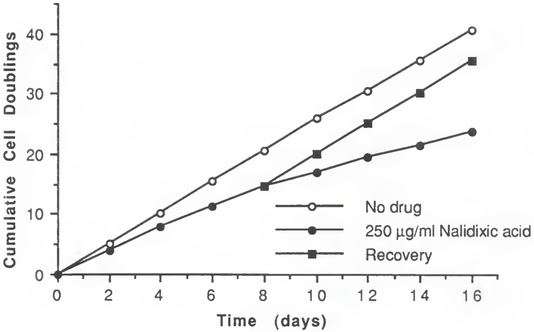
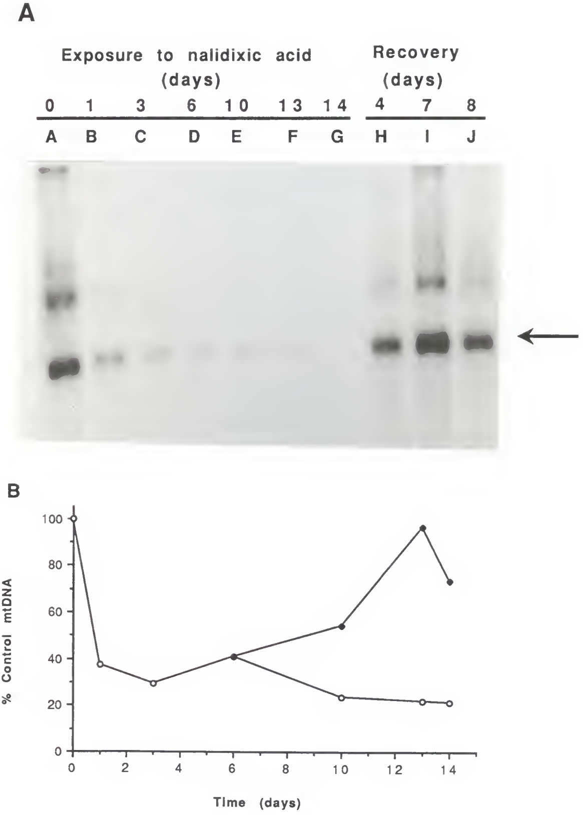


Figure 3-1 -Growth of Mouse L1210 Cells During Continuous Exposure to Nalidixic acid

Mouse L1210 cells were continuously incubated in the presence (●) or absence (○) of 1 mM nalidixic acid. At each time point, cell number was assessed using a Coulter Counter model Zf and reseeded at a density of 1 x 10^5 cells/ml in fresh media. After 6 days (arrow) of drug exposure, an aliquot (■) was washed and allowed to incubate in drug-free media to monitor recovery of growth.

Figure 3-2 -Selective Loss and Recovery of Mitochondrial DNA in Mouse L1210 Cells During Continuous Exposure to Nalidixic acid

Mouse L1210 cells were maintained at a density of 1-5 X 10^5 cells/ml in the presence of 250 µg/ml nalidixic acid. At each time point, cells were harvested and total cellular DNA was isolated as described in MATERIALS AND METHODS. After 6 days of drug exposure an aliquot of cells were washed and maintained in drug-free media to monitor recovery. Equal amounts (5 µg) of DNA were then electrophoresed for 50 hr at 70V in a 0.7% agarose gel and the mitochondrial DNA assessed by Southern blotting with the complete mouse mitochondrial DNA cloned into pSP64. The mitochondrial DNA signal was then quantified directed from the filter using a Betagen Betascope 603 Blot Analyzer and corrected for any differences in the total amount of DNA analyzed in each sample by a second hybridization to a nuclear DNA specific probe.
(A) Autoradiogram of the mitochondrial DNA content of L1210 cells treated with nalidixic acid. The arrow indicates supercoiled mitochondrial DNA.
(B) Quantification of mitochondrial DNA content of L1210 cells treated with nalidixic acid. (○)- cells incubated continuously in the presence of 250 µg/ml nalidixic acid. (●)- cells incubated in the presence of 250 µg/ml nalidixic acid for 6 days and then the drug was removed (arrow) and incubations were continued in drug-free media for an additional 8 days. Data are expressed as percent of untreated control cells.


A
Exposure to nalidixic acid (days)
Recovery (days)
0 1 3 6 10 13 14
4 7 8
A B C D E F G H I J
B
% Control mtDNA
100
80
60
40
20
0
0 2 4 6 8 10 12 14
Time (days)

required between four and seven days to returned to control levels (Fig. 3-2 lanes 8-10).

Ciprofloxacin, a more potent analogue of nalidixic acid, also inhibited mouse L1210 cell growth in a time- and dose-dependent fashion (Fig. 3-3). No effect on cell growth was evident after either one day of drug exposure at all drug concentrations employed or at concentrations up to 20 µg/ml ciprofloxacin for 7 days of drug exposure. Ciprofloxacin at 40 µg/ml inhibited cell growth dramatically after 1 day of drug exposure and 80 µg/ml ciprofloxacin inhibited cell growth by greater than 90%.

To assess the effects of ciprofloxacin on mtDNA content, total cellular DNA was isolated from mouse L1210 cells that were treated for four days with concentrations of ciprofloxacin between 20 and 100 µg/ml (Fig. 3-4). An extremely steep dose response was evident with a calculated IC_{50} value of 30 µg/ml. Ciprofloxacin at 20 µg/ml produced a reduction of less than a 20% in the mtDNA content. However, concentrations over 60 µg/ml did lead to a reduction of greater than 90% of the mtDNA-specific signal.

To examine effects of ciprofloxacin on nuclear DNA, flow cytometric analyses were performed. Figure 3-5 contains a series of histograms from cells treated with ciprofloxacin (80 or 160 µg/ml) for either one or three days before cells were fixed with ethanol and stained with propidium iodide. Ciprofloxacin had little or no effect upon cell cycle distribution after exposure to 80 µg/ml ciprofloxacin for one day. Ciprofloxacin at a concentration of 160 µg/ml did, however, show a slight increase in cells at G2/M. A slight increase (30%) in cells at G2/M became evident at ciprofloxacin concentrations of 80 µg/ml for three days. An increase in the number of cells at S phase (80% increase)

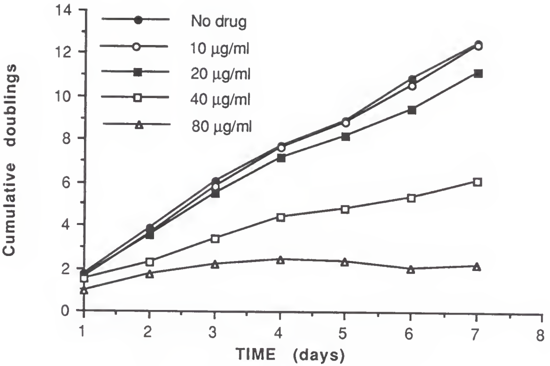


Figure 3-3 -Growth of Mouse L1210 cells in the Presence of Various Concentrations of Ciprofloxacin

Mouse L1210 cells were maintained in the presence of various concentrations of ciprofloxacin for 7 days. Each day the cell number was assessed using a Coulter Counter model Zf and the cells reseeded at a density of 1 X 10^5 cells/ml in fresh drug containing the appropriate concentration of drug. Data are expressed as cumulative cell doublings. (●)- untreated control cells; (○) 10 μg/ml ciprofloxacin; (■)- 20 μg/ml ciprofloxacin; (□)- 40 μg/ml ciprofloxacin; (Δ)- 80 μg/ml ciprofloxacin.

Figure 3-4 -Dose Dependent Loss of Mitochondrial DNA During Exposure to Various Concentrations of Ciprofloxacin

Mouse L1210 cells were maintained in the presence of 0, 20, 40, 60, 80, or 100 μg/ml ciprofloxacin for 4 days, after which the cells were harvested and total cellular DNA isolated as described in MATERIALS AND METHODS. Equal amounts (5 μg) were electrophoresed for 50 hr at 70 volts in a 0.7% agarose gel and the mitochondrial DNA content analyzed by Southern blotting with the mitochondrial DNA subclone pMBS2.1. The mitochondrial DNA was then directly quantified from the filter using a Betagen Betascope 603 Blot Analyzer and the data corrected for any differences in the total amount of DNA in each sample by a second hybridization to a nuclear DNA specific probe. Data are expressed as percent of untreated control cells. Inset: Autoradiogram of Southern blot.

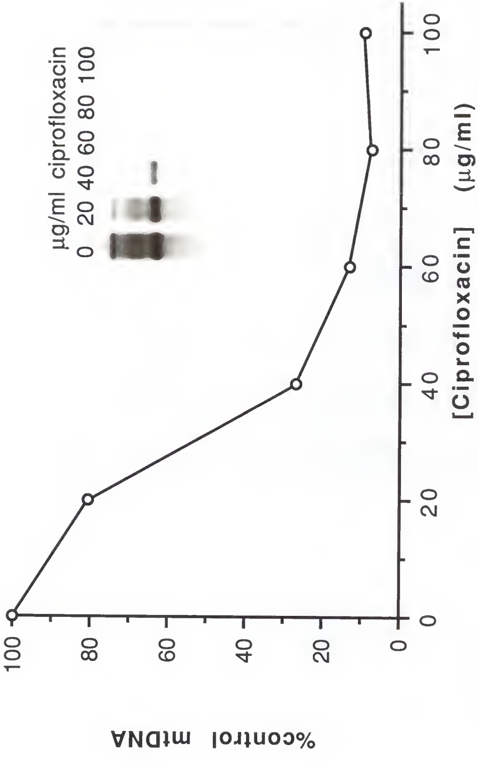
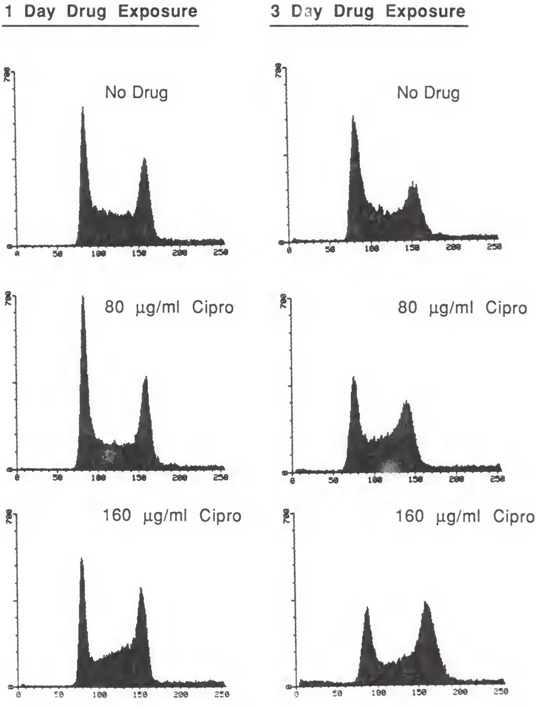

µg/ml ciprofloxacin
0 20 40 60 80 100
%control mtDNA
100
80
60
40
20
0
0
20
40
60
80
100
[Ciprofloxacin] (µg/ml)

Figure 3-5 - FACS Analysis of Mouse L1210 Cells Treated with Ciprofloxacin

Mouse L1210 cells were grown in the presence of 80 or 160 μg/ml ciprofloxacin for either 1 or 3 days. At each time point 1 x 10^6 cells were pelleted and washed in phosphate buffered saline before being fixed in ice cold ethanol as described in MATERIALS AND METHODS. FACS analysis was performed at the University of Florida Flow Cytometry Core Facility. 20,000 events were recorded and representative histograms of the cell cycle distributions are pictured above.


1 Day Drug Exposure
3 Day Drug Exposure
No Drug
No Drug
80 µg/ml Cipro
80 µg/ml Cipro
160 µg/ml Cipro
160 µg/ml Cipro
700
0
0
50
100
150
200
250

occurred at ciprofloxacin concentrations of 160 μg/ml for three days. To establish whether or not ciprofloxacin affected nuclear DNA content, an average fluorescent intensity per cell was measured in three different samples (Table 3-1). No change in DNA content was seen after 3 days of drug exposure to 80 μg/ml ciprofloxacin. However, a slight (10-20%) increase was seen at 160 μg/ml ciprofloxacin and was found to be statistically significant ($p<0.05$), but a 10% increase in nuclear DNA content could not account for a 90% decrease in mtDNA content.

Other compounds have been previously shown to cause a loss of mtDNA in mammalian cells including ethidium bromide, MGBG, and ditercalinium. Table 3-2 contains IC_{50} values of a variety of topoisomerase II active agents for both mtDNA loss and cell growth. Many agents were found to deplete cells of their mtDNA and had IC_{50} values for mtDNA loss close to their IC_{50} values for cell growth. These agents (mtDNA loss IC_{50} vs cell growth IC_{50}) included nalidixic acid (125 μg/ml vs 250 μg/ml), ciprofloxacin (80 μg/ml vs 80 μg/ml), pentamindine (300 ng/ml vs 300 ng/ml), Hoescht H33258 (1 μg/ml vs 1 μg/ml), ethidium bromide (20 ng/ml vs 20 ng/ml), and dequalinium (40 nM vs 40 nM). Other topoisomerase II agents (VM-26, adriamycin, daunomycin, and o-AMSA) had no effect on mtDNA content. Pentamidine, Hoescht H33258, ethidium bromide, adriamycin, and o-AMSA were also analyzed for mitochondrial function. All of these compounds except o-AMSA had depressed oxygen consumption rates (data not shown). With the exception of adriamycin, concentrations of drug which inhibited mitochondrial respiration were similar to those which inhibited cells growth and caused a loss of mtDNA. Although adriamycin produced a loss in oxidative function, this agent did not cause a loss of mtDNA at the concentrations tested.

Table 3-1 -FACS Analysis of Mouse L1210 Cells Treated with Ciprofloxacin

Days of Exposure [Ciprofloxacin]	Mean Fluorescence/Cell (Average ± SE)
1 Day	
No Drug	115.08 ± 4.1
80 µg/ml	120.87 ± 2.5
160 µg/ml	118.36 ± 1.0
3 Days	
No Drug	110.20 ± 9.8
80 µg/ml	103.56 ± 5.9
160 µg/ml	131.40 ± 2.4 *

FACS analysis was performed at the University of Florida Flow Cytometry Core Facility. Ethanol fixed mouse L1210 cells stained with 25 µg/ml propidium iodide after either 1 or 3 days of continuous drug exposure to either 0, 80, or 160 µg/ml ciprofloxacin were analyzed for cell cycle distribution and mean fluorescence/cell. 20,000 events were recorded and the mean fluorescence was calculated from three separate samples and the data expressed as mean ± standard error.

* $p< 0.05$

Table 3-2 -Effects of Topoisomerase II Active Agents on Cell Growth and Mitochondrial DNA Content

Drug	IC_{50} mtDNA loss	IC_{50} Cell Growth
Quinolones (Gyrase):		
Nalidixic Acid	125 µg/ml	250 µg/ml
Ciprofloxacin	30 µg/ml	30 µg/ml
Minor Groove DNA Binders:		
Pentamidine	300 ng/ml	250 ng/ml
Hoescht H33258	1 µg/ml	1 µg/ml
DNA Intercalators:		
Ethidium Bromide	20 ng/ml	20 ng/ml
Dequalinium	40 nM	40 nM
o-AMSA	NE[a]	1 µM
Daunomycin	NE[b]	30 nM
Adriamycin	NE[c]	10 nM
Nuclear Topo II inhibitors:		
VM-26	NE[d]	5 nM

IC_{50} values for mtDNA content and cell growth were based on a 4 day drug exposure regimen. L1210 Cell growth was measured by Coulter Counter and mitochondrial DNA content was assessed by analyzing total cellular DNA by Southern blotting using the mitochondrial DNA subclone pMBS2.1 as described in MATERIALS AND METHODS.

NE- No effect on mtDNA content

[a]- o-AMSA at concentrations between 0.1-1 µM

[b]- Daunomycin at concentrations between 3-30 nM

[c]- Adriamycin at concentrations between 3-30 nM

[d]- VM-26 at concentrations between 0.2-20 nM

Discussion

Mitochondrial DNA is maintained at a relatively large copy number inside cells (between 1000 and 2000 copies), and is replicated throughout the cell cycle (Bogenhagen and Clayton, 1974; Bogenhagen and Clayton, 1977). Inhibition of one or more steps in mtDNA synthesis would result in dilution of mtDNA in cells at each successive cellular division. Nalidixic acid was found to produce a selective loss of mtDNA from mammalian cells. If nalidixic acid affected a specific target involved in mtDNA synthesis, analogues of nalidixic acid should also deplete cells of their mtDNA. Ciprofloxacin, the most potent antibacterial 4-quinolone, was also shown to deplete cells of their mtDNA at concentrations approximately 10 times lower than those required for nalidixic acid. Ciprofloxacin is approximately 5-100 times more potent in its antibacterial effects than nalidixic acid (Caekenberghe and Pattyn, 1984), suggesting that the target of these agents is similar in mammalian and bacterial cells. However, effects on mtDNA content and mammalian cell growth were produced at concentrations that are much higher (greater than 50-fold) than those used clinically (Smith, 1986). Mitochondrial DNA content could decrease in two ways. Reduction could be accomplished by selectively inhibiting further mtDNA synthesis thereby diluting mtDNA at each successive cellular division or by a selective drug induced degradation of mtDNA by a nuclease. The effects of nalidixic acid on mtDNA content were not immediate, because cells treated with nalidixic acid or ciprofloxacin for 3 hours displayed no effect on mtDNA content (data not shown), suggesting that these drugs selectively inhibited mtDNA synthesis. The apparent change in mtDNA content could also occur if the nuclear DNA content increased. To ensure that loss of mtDNA from cells was not due to an increase in nuclear DNA content, flow cytometric analysis was performed. Nuclear DNA accounts for over 99% of the DNA in mammalian cells, therefore measuring total DNA per cell would measure nuclear DNA per cell. FACS analysis determined that the average DNA content per cell did not change significantly enough to account for a loss of 80-90% of the mtDNA during drug exposure.

The loss of mtDNA was accompanied by a comparable decrease in mitochondrial function, as monitored by oxygen consumption and cytochrome c oxidase activity, and was followed by an inhibition of cell growth (Lawrence et al., 1992). Oxidative phosphorylation is a major source of energy production in mammalian cells (Zeilke et al., 1984). Lactate production is associated with glycolytic energy production. Furthermore, slowing of cell growth was associated with an increase in lactate production, suggesting that these cells turned to glycolytic pathways for energy production. Since mtDNA encodes proteins involved in oxidative phosphorylation, it is not surprising that an agent which depletes mtDNA from cells also inhibits this energy generating system, thus stimulating alternative energy production pathways.

Inhibition of mtDNA content, mitochondrial function, and cell growth was found to be reversible upon drug removal with the recovery in mtDNA content and mitochondrial function preceding the recovery of cell growth. These results suggest that the slowing of cell growth induced by the 4-quinolones is a consequence of the decrease in mtDNA levels.

Previously, Ter Schegget and Borst (1971) demonstrated that nalidixic acid inhibited incorporation of nucleotides into the mtDNA of isolated chicken-liver mitochondria. Also, Mahler et al. (1968), Luha, et al. (1971), and Mahler and Johnson (1979) have shown that nalidixic acid inhibited mitochondrial DNA synthesis, mitochondrial gene expression, and mitochondrial function in yeast cells, suggesting that this mitochondrial effect of the 4-quinolones spans across many types of eukaryotic cells. Oxolinic acid, did not affect yeast mitochondrial function (Mahler and Johnson, 1979). This lack of action may be due to differences in drug permeabilities of this drug into yeast cells or mitochondria.

Castora et al. (1983) have studied the effects of antibacterial agents on mammalian mtDNA synthesis. These results showed that mtDNA replication in isolated mitochondria could be arrested by the DNA gyrase inhibitors nalidixic acid and oxolinic acid. It has been postulated that mitochondria contain a DNA gyrase-like (bacterial type II topoisomerase) enzyme to supercoil and decatenate mtDNA during initiation and termination steps in mtDNA replication (Clayton, 1982, Castora et al., 1982). However, experiments performed in isolated mitochondria by Riesbeck et al. (1990) did not support these results. The authors reported that ciprofloxacin did not inhibit mitochondrial DNA synthesis in isolated mitochondria. This was in complete contrast to the study by Castora et al. (1983) in which they reported that two other 4-quinolones inhibited nucleotide incorporation into supercoiled mtDNA circles but not 9S initiator strand molecules, suggesting that these agents inhibited elongation and not initiation of mtDNA. It is possible that Riesbeck et al. (1990) were examining the effects of ciprofloxacin on 9S initiator strand synthesis, and this would not allow any differences in elongation to be measured. Also, the authors could not explain why ciprofloxacin did not act like other 4-quinolones that were shown to inhibit mtDNA synthesis in isolated mitochondria (Castora et al., 1983). A likely reason why Reisbeck et al. (1990) did not see any effect on mtDNA replication is that ciprofloxacin is concentrated inside the cell up to 10 times the extracellular concentration (Easmon and Crane, 1985). In Riesbeck et al. (1990), the authors were using concentrations of ciprofloxacin that did not reflect the intracellular concentrations.

Several different drug classes have been shown to deplete mammalian cells of their mtDNA, including the 4-quinolones (nalidixic acid and ciprofloxacin), intercalators (ethidium bromide and dequalinium), and the minor groove DNA binders (pentamidine and Hoescht H33258). Of these drugs, ethidium bromide and pentamidine have recently been shown to interact with a mitochondrial type II topoisomerase in trypanosomes (Shapiro and Englund, 1990). Dequalinium, a compound used as an antimicrobial agent for many years, has been shown to be accumulated in mitochondria and have significant antitumor activity (Weiss et al., 1987), but the mechanism of action for this drug is not understood.

Nuclear topoisomerase II inhibitors m-AMSA, VM-26, and adriamycin did not induce a loss of mtDNA from mammalian cells. These nuclear topoisomerase II drugs have been shown to be cytotoxic to actively dividing cells. Furthermore, Lin and Castora (1991) have reported that a partially purified mitochondrial topoisomerase II-like activity was more sensitive to the inhibitory effects of m-AMSA and VM-26. One possible reason why they do not deplete mtDNA from cells is that they produce such strong nuclear effects that cells die before they lose mtDNA. Unlike nuclear DNA, mtDNA is continuously turned over with a half-life of approximately 1 week (Gross et al., 1969). If cells no longer divided, the nuclear component of these drugs may be eliminated and possibly a mtDNA effect could be seen. Perhaps if stationary phase cells were cultured for long periods of time with m-AMSA, VM-26, or adrimycin, these drugs would deplete mtDNA form cells, because unlike nuclear DNA, mtDNA continually turns over (Gross et al., 1969).

It may not be that surprising that mitochondria are affected by the 4-quinolones simply because many antibacterial agents also have effects on mitochondrial proteins and processes. Chloramphenicol and tertacycline inhibit protein synthesis in both bacteria and mitochondria (Van Den Borgert and Kroon, 1981). The endosymbiotic theory states that mitochondria were derived from bacterial origins (Gray, 1989). It is therefore not entirely suprising that inhibitors of bacterial topoisomerase II enzymes affect mitochondrial DNA metabolism.

One likely target of nalidixic acid and ciprofloxacin is a mitochondrially located type II topoisomerase-like enzyme. Castora et al.(1983) believed mitochondria to contain a DNA gyrase-like enzyme because of the inhibitory effects of nalidixic acid and oxolinic acid on mtDNA synthesis. Circular DNA molecules such as mtDNA are dependent upon topoisomerase II enzymes to relieve topological problems that arise during replication and to decatenate the two newly replicated interlocked DNA molecules (Drlica et al., 1977; Clayton, 1982; Castora et al., 1982; and Berk and Clayton, 1976). Melendy and Ray (1989) purified a mitochondrial topoisomerase II activity from the trypansomatid Crithidia fasciculata. This activity was inhibited by concentrations of nalidixic acid that are effective against the bacterial enzyme DNA gyrase. This mitochondrial type II topoisomerase was found to have physical and antigenic properties that were distinct from the nuclear type II topoisomerase in this organism.

Although a type II topoisomerase is a likely target for drug action, other activities are also required for mtDNA replication (ie. DNA polymerases, helicases, etc.) that may be inhibited by 4-quinolones. Drugs that are structurally unrelated to 4-quinolones, such as the DNA intercalator ethidium bromide, the polyamine analogue MGBG, and the dideoxynucleoside analogue ddC, have also been shown to deplete mammalian cells of their mtDNA (Wiseman and Attardi, 1978; Nass, 1984; Chen and Cheng, 1989). The mechanisms underlying depletion of mtDNA by ethidium bromide and MGBG are unknown. However, there is some evidence suggesting that ddC may be acting by inhibiting the mitochondrial enzyme DNA polymerase gamma (Chen and Cheng, 1989). However, in vitro studies suggest that this enzyme is not inhibited by the 4-quinolone drugs nalidixic acid or oxolinic acid (Castora et al., 1983). Further analysis as to whether or not the 4-quinolones interact with a type II topoisomerase in mitochondria are presented and discusses in Chapter 5 of this dissertation.

CHAPTER 4

CYTOTOXIC EFFECTS OF CIPROFLOXACIN

Introduction

Previous work has demonstrated that ciprofloxacin depleted cells of mtDNA (see Chapter 3 of this dissertation). Mitochondrial depletion was associated with a loss of mitochondrial function and slowing of cell growth. Hussy et al. (1986) have also shown that ciprofloxacin inhibited cell growth. The apparent reversible effects of nalidixic acid on mtDNA content, mitochondrial function, and cell growth suggested that this drug was cytostatic but not cytotoxic. Ciprofloxacin inhibits bone marrow engraftment in mice, suggesting that there may be a cytotoxic component to ciprofloxacin's growth inhibitory actions (Somekh et al., 1989). In addition, 4-quinolones have demonstrated activity in treatment of hyperproliferative skin disorders (Bohr et al., 1987). The underlying mechanisms involved in the antiproliferative action of 4-quinolones is not well understood. However, it has been proposed that 4-quinolones may be acting through a mitochondrial DNA topoisomerase II-like enzyme that affects mtDNA synthesis (Castora et al., 1983). Our goal was to determine if ciprofloxacin's cytotoxic effect was mediated through a mitochondrial mechanism. This goal was accomplished by determining if cells grown under respiration-independent conditions are resistant to the cytotoxic effects of ciprofloxacin.

Results

L1210 cell viability was measured at various times of exposure to ciprofloxacin (100 μg/ml). Viable cells normally exclude trypan blue by an active efflux mechanism (Roper and Drewinko, 1976). Viability was determined by dividing the number of cells that excluded trypan blue by the total number of cells. As can be seen in Figure 4-1, cell viability remained unchanged for two days of drug exposure. After two days, cell viability (as measured by trypan blue uptake) steadily decreased to 70% of control cells by day six. Viability in untreated cells remained at virtually 100% throughout the experiment.

Trypan blue exclusion is not a good measure of cytotoxicity because it does not always correlate with cell death (Roper and Drewinko, 1976). Therefore, clonogenic cytotoxicity experiments were employed to verify results obatined by trypan blue exclusion. Clonogenic cytotoxicity assays measure the ability of single cells to grow into colonies. In Figure 4-2, mouse B16 cells were plated out into 6-well tissue culture plates and allowed to attach overnight. Media was then removed and replaced with fresh media containing desired concentration of ciprofloxacin. Cells were then incubated for various periods of time. Cells were then washed free of the drug and allowed to incubate for six more days in fresh, drug-free media, allowing any viable cells to grow into colonies. Ciprofloxacin had no effect on B16 cell growth at any concentration tested for periods of time up to one day. However, a decreased cloning efficiency was observed at drug concentrations greater than 60 μg/ml. A maximal 1.5 log kill was observed at a ciprofloxacin concentration of 120 μg/ml during drug exposures that lasted 6 days. HeLa cells displayed a similar cytotoxic profile (Figure 4-3). Ciprofloxacin had no effect on HeLa cell growth at any concentration tested for periods of time up to 2 days. After 2 days of

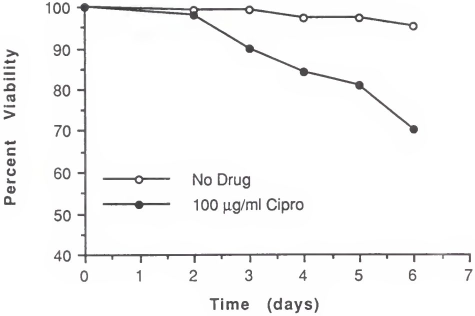


Figure 4-1 -Effect of Ciprofloxacin on Mouse L1210 Cell Viability

L1210 cells were seeded into a 150 cm^2 tissue culture flask and the cells counted each day by hemacytometer as described in MATERIALS AND METHODS. Data are expressed as the percent to total cells counted that stain positive for trypan blue. (○)- untreated cells; (●)- cells continuously grown in the presence of 100 µg/ml ciprofloxacin.

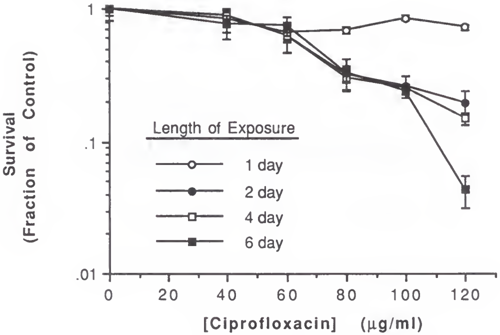


Figure 4-2 -Cytotoxicity of Ciprofloxacin to Mouse B16 melanoma Cells

The cytotoxiticity of ciprofloxacin was assessed using a clonogenic assay as described in MATERIALS AND METHODS. (○)- cells treated for 1 day (●)- cells treated for 2 days (❑)- cells treated for 4 days (■) cells treated for 6 days. Samples were performed in triplicate and the data plotted as fraction of control ± standard error.

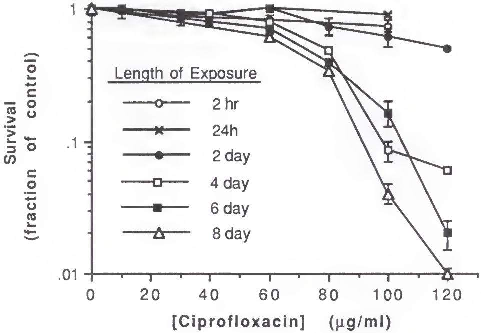


Figure 4-3 -Cytotoxicity of Ciprofloxacin to Human HeLa Cells

The cytotoxiticity of ciprofloxacin was assessed using a clonogenic assay as described in MATERIALS AND METHODS. (○)- cells treated for 2 hr (X)- cells treated for 1 day (●)- cells treated for 2 days (❑)- cells treated for 4 days (■) cells treated for 6 days (Δ)- cells treated for 8 days. Samples were performed in triplicate and the data plotted as fraction of control ± standard error.

ciprofloxacin exposure, a decreased cloning efficiency was observed at drug concentrations greater than 60 μg/ml. A maximal 2 log kill was observed at a ciprofloxacin concentration of 120 μg/ml during a drug exposures that lasted 8 days.

Several anticancer agents are thought to kill cells through the induction of enzyme-DNA complexes in nuclear DNA (Nelson et al., 1984; Tewey et al., 1984b; Ross et al., 1984). Ciprofloxacin targets DNA gyrase (bacterial topoisomerase II), suggesting that, at high enough concentrations, ciproflxoacin may interact with a eukaryotic nuclear topoisomerase II. Induction of nuclear DNA topoisomerase II mediated cleavable-complexes may be the cytotoxic mechanism of ciprofloxacin. To test this hypothesis, cytotoxic effects of ciprofloxacin to VPM^{R}5 cells were assessed using a clonogenic cytotoxicity assay. VPM^{R}5 cells are a CHO cell line contining a single amino acid change in the topoisomerase II enzyme that makes it 8- to 10-fold more resistant to nuclear topoisomerase II drugs. Ciprofloxacin was found to be cytotoxic to VPM^{R}5 cells in a dose dependent fashion at the same concentration range that killed wild type CHO cells (Figure 4-4), suggesting that nuclear DNA topoisomerase II mediated DNA damage was not the cytotoxic mechansim of ciprofloxacin. However, ciprofloxacin may not interact at the same site of the topoisomerase II enzyme as VM-26, and, therefore, a direct analysis of nuclear cleavable-complex formation is warranted.

It has been previously shown that ciprofloxacin induced nuclear topoisomerase II mediated DNA breaks (Bredberg et al., 1991) To determine if ciprofloxacin induced significant nuclear cleavable-complex formation, a glass binding assay was performed. In this assay, glass specifically binds protein in the presence 0.4 M guanidine-HCl and 0.3 M NaCl (Shin and Snapka, 1990). Therefore, only DNA that is attached covalently to protein will bind to glass

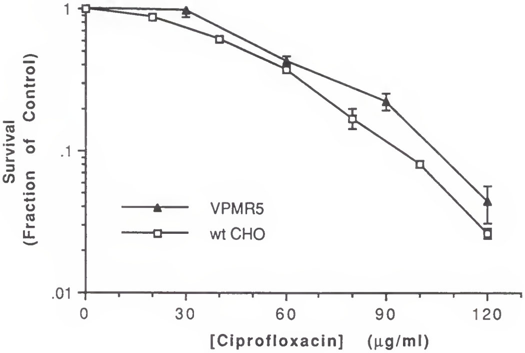


Figure 4-4 -Cytotoxicity of Ciprofloxacin to VPM^{R}5 Cells

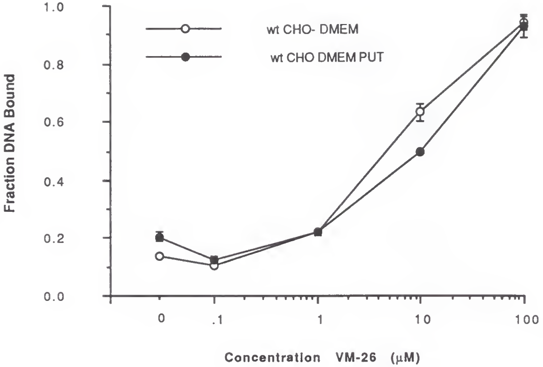
The cytotoxiticity of ciprofloxacin was assessed using a clonogenic assay which tested the ability of single cells to grow into colonies during drug exposure. VPM^{R}5 cells, a CHO subline resistant to the anticancer agents VP-16 and VM-26, or wild type CHO cells were plated out in α-MEM media and allowed to attach overnight as described in MATERIALS AND METHODS. (❑) wild type CHO cells treated for 4 days; (▲)- VPM^{R}5 cells treated for 4 days. Samples were run in triplicate and the data plotted as fraction of control ± standard error.

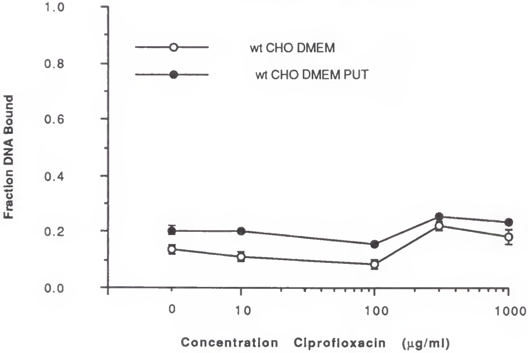
under these conditions. Wild type CHO cells were prelabeled with ^{3}H-thymidine overnight before they are washed free of label and incubated in fresh, drug-free media for another two hours at 37°C. Cells were then incubated with various concentrations of either VM-26, a specific nuclear type II topoisomerase inhibitor, or ciprofloxacin. Cells were pelleted, lysed, and filtered through GF/C glass filters under conditions in which only protein would bind to the glass. In panel A of figure 4-5, it is evident that VM-26 produced a dose-dependent increase the amount DNA that bound to the glass filters. In fact, concentrations of 100 µM VM-26 bound all labeled DNA to the glass filters. This increase in glass filter binding is consistent with formation of DNA-topoisomerase II complexes. Panel B of figure 4-5 demonstrates that ciprofloxacin had little effect on the amount of DNA binding to glass filters. A small increase in DNA binding was produced at concentrations over 100 µg/ml, but were not nearly the magnitude of those produced by VM-26.

These data suggested that ciprofloxacin induced cytotoxicity was not mediated through a nuclear DNA topoisomerase II mechanism. Therefore, an alternative cytotoxic mechanism involving the loss of mtDNA was investigated. Other groups have developed cell lines that grow in the absence of mtDNA and mitochondrial function (King and Attardi, 1989; Desjardins et al., 1986). These cell lines continue to grow because the requirement for mitochondrial function has been by-passed by supplementing the growth media with glucose, pyruvate, and pyrimidines. If ciprofloxacin is acting by interfering with mitochondrial function, then these cells no longer containing mtDNA or not requiring mitochondrial function should be resistant to this drug. We therefore set out to isolate cell lines devoid of mtDNA to test our hypothesis that ciprofloxacin killed cells through a mitochodnrial mechansim. The rationale for

Figure 4-5-Nuclear Cleavable-Complex Formation in CHO Cells After Exposure to VM-26 or Ciprofloxacin

Wild type CHO cells in DMEM (○) or DMEM PUT (●) media were labeled with 0.5 µC/ml ^{3}H-thymidine for 24 hrs before the cells were washed free of the label and incubated for 2-3 hrs in fresh label-free media to remove unincorporated label. Equal numbers of cells were then incubated in 4 ml of fresh drug containing media for 1 hr before the cells were pelleted and the supernate discarded. The samples were then lysed with 200 µl of 20 mM Tris pH 8.0, 1% SDS, 1 mM PMSF before the samples were filtered through glass fiber filters under conditions specifically designed to bind only protein-linked DNA (see MATERIALS AND METHODS). Each sample was performed in triplicate and the data expressed as fraction of total counts ± standard error.
(A) Cells treated with VM-26.
(B) Cells treated with ciprofloxacin.


1.0
0.8
0.6
0.4
0.2
0.0
Fraction DNA Bound
wt CHO- DMEM
wt CHO DMEM PUT
0
.1
1
10
100
Concentration VM-26 (μM)

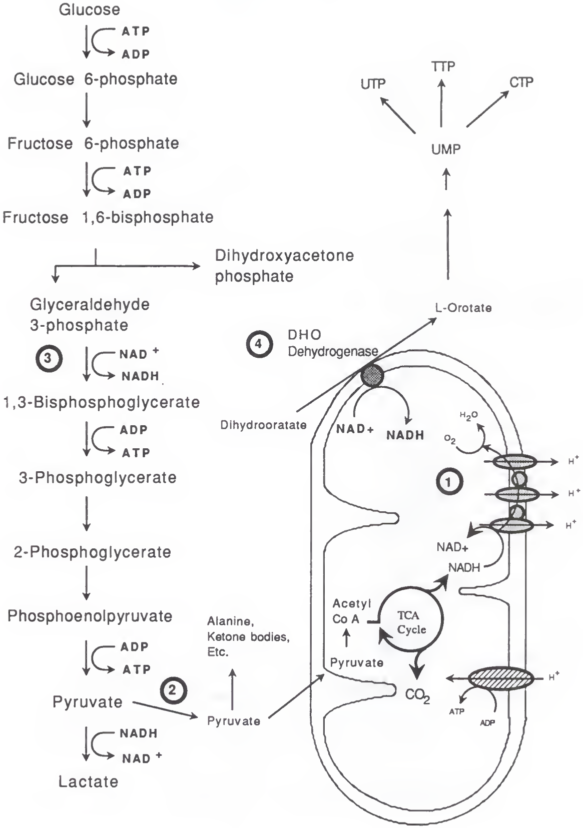

1.0
0.8
0.6
0.4
0.2
0.0
Fraction DNA Bound
wt CHO DMEM
wt CHO DMEM PUT
0
10
100
1000
Concentration Ciprofloxacin (μg/ml)

developing a mtDNA minus cell line is outlined in Figure 4-6. Mitochondria are the organelles that are responsible for producing a great portion of the cells energy through the process of oxidative phosphorylation (see ①, Figure 4-6). Alternatively, cells can generate energy through glycolysis.

De novo pyrimidine biosynthesis is also dependent on mitochodnrial function. Dihydroorotate dehydrogenase catalyzes the conversion of dihydrooratate to L-orotate, a step in pyrimidine nucleotide biosynthesis. In mammalian cells, this enzyme's activity is coupled to the NADH to NAD^+ redox cycle in the electron chain transport system as a mechanism to remove the electrons it receives from dihydroorotate (Gregoire et al., 1984; Loffler, 1980; Miller et al., 1968). Consequently, mammalian DHO dehydrogenase has become dependent on active mitochondrial respiration in order to regenerate its reduced form and then allow it to accept the electrons from another dihydrooratate molecule (see ④, figure 4-6). Therefore, in the absence of DHO dehydrogenase activity, supplementing the growth media of cells with uridine (a pyrimidine) would by-pass the need for for this respiration-dependent enzyme.

When mitochondrial function is absent, pyruvate metabolism is affected. Glyceraldehyde 3-phosphate dehydrogenase requires NAD^+ as an electron acceptor in its reaction (see ③, figure 4-6). Lactate dehydrogenase uses NADH as an electron donor forming NAD^+. In the absence of respiratory function, these two enzymes are dependent upon each other's redox cycling of NAD^+. This forces all of the pyruvate formed in glycolysis to be metabolized to lactate in order to regenerate the NAD^+ required in its reaction. Pyruvate is necessary as a precursor of alanine and ketone bodies (see ②, figure 4-6). Therefore, energy metabolism in the absence of respiratory function requires that pyruvate be converted to lactate, thus inhibiting the synthesis of amino acids or ketone

Figure 4-6 -Biosynthetic Pathways Dependent on Oxidative Phosphorylation


Glucose
ATP
ADP
Glucose 6-phosphate
Fructose 6-phosphate
ATP
ADP
Fructose 1,6-bisphosphate
Dihydroxyacetone phosphate
Glyceraldehyde 3-phosphate
③
NAD +
NADH
1,3-Bisphosphoglycerate
ADP
ATP
3-Phosphoglycerate
2-Phosphoglycerate
Phosphoenolpyruvate
ADP
ATP
Pyruvate
②
NADH
NAD +
Lactate
Alanine, Ketone bodies, Etc.
Pyruvate
TTP
UTP
CTP
UMP
L-Orotate
④
DHO Dehydrogenase
Dihydroorotate
NAD+
NADH
H_2O
O_2
H^+
H^+
H^+
①
NAD+
NADH
Acetyl Co A
TCA Cycle
Pyruvate
CO_2
H^+
ATP
ADP

bodies. Hence, in the presence of glucose, pyruvate, and uridine (a pyrimidine), cells theoretically could survive without mtDNA. Other groups have developed cell lines lacking mtDNA (a chicken fibroblast cell line and a HeLa cell line) by incubating them with ethidium bromide in growth media containing glucose, pyruvate, and uridine (Desjardins et al., 1986; King and Attardi, 1989). Ethidium bromide is a DNA intercalating agent that has been used to cure plasmids from bacteria and mtDNA from mammalian cells (Waring, 1975; Wiseman and Attardi, 1978).

We were unsuccessful at isolating a mtDNA minus cell line from HeLa, L1210, or B16 cells. However, CHO cells eventually did grow in the presence of ethidium bromide (0.4 µg/ml) in DMEM media containing 2x MEM amino acids, 2x MEM vitamins, 4.5 g/l glucose, 1 mM pyruvate, 10 µg/ml uridine, and 10 µg/ml thymidine (designated DMEM PUT media). In the beginning of the selection, CHO cell growth slowed down after 8 days of incubation with ethidium bromide (0.4 µg/ml). After 19 days, cell growth resumed, and continued thereafter. After 25 days of growth in the continuous presence of ethidium bromide (0.4µg/ml), several CHO cell lines derived from single cells were grown using a dilution method for cloning (designated as CHOE cell lines). In Figure 4-7, 8 individual isolates were grown up and analyzed for cytochrome c oxidase activity (COX). Recall that cytochrome c oxidase has three subunits encoded for by mtDNA. COX activity was depressed in two of eight of the cell lines. These two cell lines were then grown in the presence of ethidium bromide for another 2 weeks (designated as CHOE3 and CHOE4 cell lines).

After 2 weeks of ethidium exposure, another analysis of COX activity was performed on the CHOE3 and CHOE4 cell lines (Figure 4-8). CHOE3 cells

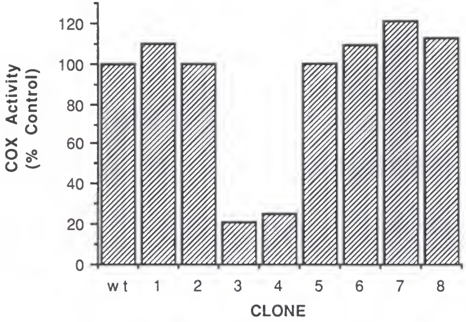


Figure 4-7 -Cytochrome C Oxidase Analysis of Ethidium Bromide Resistant Cell Lines

Cell clones resistant to ethidium bromide (0.4 µg/ml) were selected by first maintaining a population of CHO cells in DMEM PUT media for 1 month. Selection of clones arising from a single cell were obtained by diluting 70 cells into a 96 well tissue culture plate and selecting wells which contained one colony as determined by light microscopy. These colonies were detached as described in MATERIALS AND METHODS and grown successively in 24 well and 6 well tissue culture plates and then transferred to 75 cm^2 tissue culture flasks. Cell lines were analyzed for COX activity as described in MATERIALS AND METHODS. Data are expressed as percent of wild type cells.

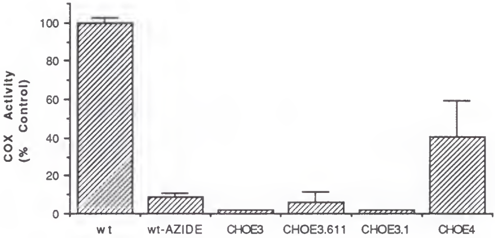


Figure 4-8 -Further Analysis of Cytochrome C Oxidase Activity of Ethidium Bromide Resistant Cell Lines

The two clones with depressed COX activities (CHOE3 and CHOE4) were either placed in ethidium-free media and passaged for 2 weeks, passaged in media containing 1 µg/ml ethidium bromide for 2 weeks, or passaged in 0.4 µg/ml ethidium bromide for 2 weeks before analyzing for COX activity as described in MATERIALS AND METHODS. Also included were wild type control cells treated with 1 mM sodium azide during the COX assay. Data are expressed as percent of wild type cells ± standard error.

retained low COX activity levels (3% of untreated wild type CHO cells). CHOE4 cells also had low COX acitivity levels, but levels were much higher (40% of untreated wild type CHO cells) than the CHOE3 cells . Wild type CHO cells treated with 1 mM sodium azide (an inhibitor of cytochrome c oxidase) during COX analysis, had a COX activity of 10% that of untreated wild type CHO cells. The residual COX actitivy remaining during sodium azide treatment is probably non-specific oxidation of cytochrome c by cell homogenate and is not related to oxidative phosphorylation. CHOE3 cells placed in 1 µg/ml ethidium bromide (designated CHOE3.1) for 2 weeks continued to have decreased levels of COX acitivity (3% of untreated wild type CHO cells). When CHOE3 cells were placed in ethidium-free media (designated CHOE3.611) for two weeks, levels of COX activity remained unchanged (8% of untreated wild type CHO cells), suggesting that the loss of COX activity was a stable phenotype. These results suggest that the CHOE3 cell line contained no COX acitivity, because all COX acitivity values were below values of the sodium azide treated homogenates.

When oxygen consumption measurements were performed, the CHOE3.1 cells displayed little, if any, respiratory activity. Antimycin A, an inhibitor of complex II (between cytochromes b and c_1), did not affect the residual oxygen consumption rate measured in these cells, suggesting that this oxygen consumption was from a source other than oxidative phosphorylation. Wild type CHO cells did have their oxygen consumption rate inhibited by antimycin A (data not shown). CHOE3.1 cells did, however, regain their ability to respire if grown for over two weeks in the absence of ethidium bromide. These results suggested that these cells retained some of their mtDNA required to restore the lost COX activity upon ethidium bromide removal. Subsequently, preliminary mtDNA content analysis of these cells revealed that these cells did in fact retain some mtDNA. Ethidium bromide has been shown to inhibit mtDNA transcription, and is probabily the reason these cells have no COX activity (Bendich, A.J., 1987). It was then decided to use the CHOE3.1 cells (which had no COX activity or respiratory activity as measures of mitochondrial function) to test our hypothesis that ciprofloxacin kills cells through a mitochondrial mechanism.

A clonogenic cytotoxicity assay was used to determine the cytotoxic effects of ciprofloxacin on wild type cells in the DMEM media, alpha-MEM media, or DMEM PUT media (Figure 4-9). This was compared to the effects of ciproflxoacin on CHOE3.1 cells grown in DMEM PUT media. Table 2-1 describes components of the different media used in these experiments. DMEM media, which contains glucose but no pyruvate or pyrimidines, requires mitochondrial activity for cell survival. DMEM PUT media is supplemented with pyruvate and pyrimidines and can support cell growth in the absence of mitochondrial function. Alpha-MEM media is similar to DMEM (see GIBCO catalog for specific differences) except that it contains less glucose, has pyruvate, but contains no pyrimidines. Wild type CHO cells grown in either DMEM or alpha-MEM media displayed a dose dependent sensitivity to ciprofloxacin. In contrast, growth of CHOE3.1 cells was unaffected by ciprofloxacin. In addition, wild type CHO cells grown in DMEM PUT media displayed the same resistance to ciprofloxacin as CHOE3.1 cells, suggesting that ciprofloxacin was not cytotoxic to cells grown under conditions requiring respiratory function. In experiments in which wild type CHO cells were used without any adaptation time to DMEM PUT media, some cytotoxicity at the highest concentrations was evident (data not shown).

One possible explanation for the drug resistance in wild type CHO and CHOE3.1 cells grown in DMEM PUT media was that there was less

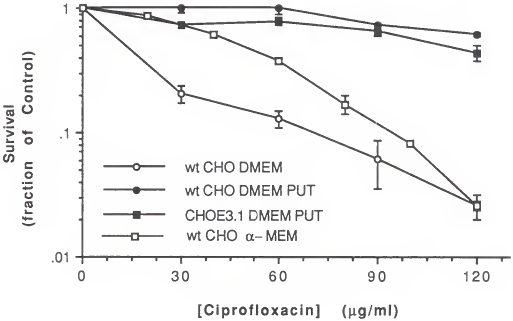


Figure 4-9 -Cytotoxic Effects of Ciprofloxacin on CHO Cells Grown in Respiration-Dependent and -Independent Conditions

The cytotoxiticity of ciprofloxacin was assessed using a clonogenic assay as described in MATERIALS AND METHODS. Wild type CHO cells in DMEM PUT media were passaged in DMEM PUT media for at least 2 days before initiation of the cytotoxicity experiment. (○)- Wild type CHO cells treated for 4 days in DMEM media; (●)- Wild type CHO cells treated for 4 days in DMEM PUT media; (☐)- Wild type CHO cells treated for 4 days in α-MEM media; (■)- CHOE3.1 cells treated for 4 days in DMEM PUT media. Samples were run in triplicate and the data plotted as fraction of control ± standard error.

accumulation of ciprofloxacin into cells. To test this possibility, a drug accumulation experiment was performed. Ciprofloxacin accumulation was measured by fluorescent spectroscopy in wild type cells and CHOE3.1 grown in alpha-MEM or DMEM PUT media, respectively. Wild type CHO cells in alpha-MEM media accumulated approximately 0.25 fg/cell (Figure 4-10). Wild type CHO cells in DMEM PUT media actually had a higher drug accumulation (0.55 fg/cell), while the mutant cell line CHOE3.1 had approximately the same accumulation as wild type cells in alpha-MEM media (0.23 fg/cell). Even though the DMEM PUT media did change the accumulation of ciprofloxacin, it produced an increase, rather than a decrease, in drug levels inside cells. These results suggest that reduced intracellular ciprofloxacin concentrations were not responsible for resistance in wild type CHO or CHOE3.1 cells grown in DMEM PUT media.

Discussion

These results suggest that ciprofloxacin's cytotoxic effect is mediated through a mechanism involving mitochondrial function, since ciprofloxacin does not affect cells grown under conditions that by-pass the requirement for mitochondrial function.

Ciprofloxacin was shown to be cytotoxic to B16 cells at concentrations greater than 60 µg/ml during drug exposures lasting more than one day. Concentrations of ciprofloxacin that kill cells are similar to those which deplete mtDNA, suggesting that drug-induced depletion of mtDNA was responsible for the loss of cell viability. Conversely, ciprofloxacin was shown to be cytotoxic to HeLa cells at conentrations greater than 60 µg/ml during drug exposures lasting

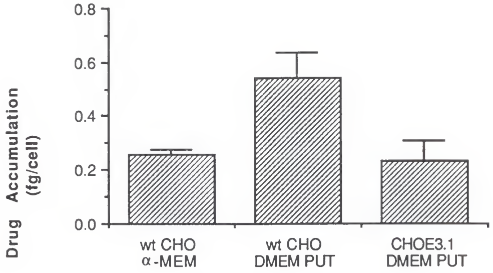


Figure 4-10 -Ciprofloxacin Accumulation in CHO Cells

Ciprofloxacin accumulated in wild type CHO and CHOE3.1 cells grown in α-MEM (wild type CHO cells) or DMEM PUT (wild type CHO and CHOE3.1 cells) was monitored by a fluorometric assay as described in MATERIALS AND METHODS. Briefly, cells (1 X 10^7) were incubated in the presence or absence of 100 µg/ml ciprofloxacin for 1 hr before washing away the extracellular drug. Nonspecific fluorescence contributed by the cell was subtracted out using the untreated control cells. Samples were compared to an external standard and data expressed as fg/cell ± standard error.

more than two days. Even though the lengths of drug treatment required to kill B16 and HeLa cells were different , the IC_{50}s for HeLa and B16 cells was similar. The time required to kill HeLa cells was twice as long as that required to kill B16 cells. HeLa cells divide every 24 hours, while B16 cells divide every 14 hours, nearly twice as fast as the HeLa cells. The time required to kill HeLa cells was twice as long as that required to kill B16 cells and HeLa cells divide twice as slow as B16 cells, suggesting that the cytotoxic effects of ciprofloxacin are related to cellular division.

It has been reported that ciprofloxacin produces double and single stranded breaks in nuclear DNA as determined by alkaline elution (Bredberg et al., 1991). In contrast to the results by Bredberg et al. (1991), we observed no significant nuclear cleavable-complex formation in CHO cells.

One possibility is that cleavable-complexes produced by VP-16 are different from those produced by ciprofloxacin. In their paper, the Bredberg et al. (1991) demonstrated that the ciprofloxacin induced DNA strand breaks reversed approximately 4 times faster than the VP-16 induced DNA strand breaks when the drug was washed out. Drug retention differences could explain the differences in ciprofloxacin and VP-16 induced cytotoxicity because VP-16 washes out of cells well and all strand breaks are reversed within 60 min of incubation in drug-free media. Another possibility is that Raji cells are more sensitive to nuclear DNA strand breaks caused by ciprofloxacin than other cell types. In fact, Raji cells contain two distinct forms of nuclear topoisomerase II enzymes having different molecular weights (Drake et al., 1989). These two forms of nuclear topoisomerases may be pharmacolgically different and display differential sensitivly to ciprofloxacin. Futhermore, we have found that ciprofloxacin does in fact stimulate nuclear cleavable-complex formation in Raji cells (data not shown). These cleavable-complexes occur at concentrations of ciprofloxacin that agree with the studies by Bredberg et al. (1991). The topoisomerase II drug resistant cell line VPM^{R}5 is sensitive to ciprofloxacin at concentrations that kill wild type CHO cells. The question then becomes: why are VPM^{R}5 cells as sensitive to ciprofloxacin as wild type CHO cells? This may be elucidated by analyzing the amounts of nuclear cleavable-complexes formed in wild type CHO cells during exposure to ciproflxoacin. Wild type CHO cells exposed to various concentrations of VM-26 as a positive control produced a dose dependent increase in protein-linked DNA, consistent with formation of nuclear topoisomerase II-DNA cleavable complexes. Ciprofloxacin, on the other hand, did not stimulate significant nuclear cleavable complex formation, except at doses over 100 μg/ml. These higher doses produced only a small increase in protein-linked DNA formation and were 5 to 10 times the concentration of ciprofloxacin required to kill cells. Therefore, nuclear cleavable complex formation is probably not involved in the cytotoxic mechanism of ciprofloxacin.

Treatment of wild type CHO or CHOE3.1 cells under conditions that support respiratory-independent growth resulted in no cytotoxicity. Drug accumulation studies demonstrated that ciprofloxacin accumulates to similar levels in cells regardless of the growth media, ruling out a drug accumulation mechanism of resistance, futher suggesting that the cytotoxic effect of ciprofloxacin is due to its effect on mtDNA. Futhermore, if nuclear DNA cleavage was important, then cells should still be sensitive when grown in DMEM PUT media because this media was shown not to significantly affect the formation of nuclear DNA cleavable complexes.

Forsgren et al (1987b) reported that ciprofloxacin inhibited mammalian cell growth and stimulated ^{3}H-thymidine incorporation into DNA. If de novo

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pyrimidine synthesis was inhibited during ciprofloxacin treatment, then cells would need to obtain their pyrimidines from another source. Stimulated ^{3}H-thymidine uptake into cells during ciprofloxacin treatment suggests that de novo pyrimidine synthesis is inhibited by this drug. Therefore, these investigators hypothesized that ciprofloxacin inhibited cell growth by interfering with pyrimidine biosynthesis. Lyons and Christopherson (1990) reported that ciprofloxacin did not have a direct effect on de novo pyrimidine synthesis or the enzymes involved in this pathway, notably dihydroorotate dehydrogenase. These studies were performed after a 4 hour drug treatment, a period of time not long enough to deplete cells of their mtDNA. If the enzyme dihydroorotate dehydrogenase was inhibited during ciprofloxacin exposure, the cells should be rescued by addition of uridine to the media (Chen et al., 1986). However, Forsgren et al. (1987b) reported that addition of uridine did not rescue the cells from ciprofloxacin's effect on cell growth and concluded that ciprofloxacin did not effect the dihydroorotate dehydrogenase enzyme in these cells.

The fact that ciprofloxacin did not affect dihydroorotate dehydrogenase directly does not exclude the possibility that a loss of mtDNA produced by ciprofloxacin could inhibit this enzyme. Attardi et al. (1989) developed a mammalian cell line devoid of mitochondrial DNA that were auxotrophic for both uridine and pyruvate, and found that cells lacking mtDNA could not survive without both of these factors. Therefore, in order to rescue cells treated with ciprofloxacin, growth media must contain both pyruvate and uridine because ciprofloxacin treated cells could not perform oxidative dependent pathways due to decreased mtDNA content. Forsgren et al. (1987b) performed their rescue experiment in DMEM media. This media did not contain pyruvate, and therefore did not support mtDNA independent growth. For this reason, ciprofloxacin treated cells in DMEM media did not survive when a rescue experiment was tried with the addition of uridine alone. Both uridine and pyruvate were required in a rescue experiment to counteract a loss of dihyroorotate dehydrogenase due to a loss of mitochondrial encoded function.

It has been reported that a minimum of 10% of the mtDNA be retained in order to support cell viability (Wiseman and Attardi, 1978). If mtDNA was diluted out of cells at each successive cellular division, it would take approximately 4 doublings before mtDNA content dropped below 10%. In both cytotoxicity experiments involving HeLa and B16 cells, drug exposures below 4 cellular doublings had no effect on cell viability. When drug exposures lasted 4 or more doublings, viability decreased. MGBG, another mtDNA depleting agent, causes a successive halving of the mtDNA content at each cellular division during drug exposure (Bortell, 1987). It is tempting to speculate that ciprofloxacin acts in a similar way. Recall, nalidixic acid treatment of cells led to an exponential loss of the mtDNA that was dependent upon several successive cellular divisions.

CHAPTER 5

IDENTIFICATION OF A DRUG SENSISTIVE TYPE II DNA TOPOISOMERASE ACTIVITY IN MAMMALIAN MITOCHONDRIA

Introduction

One likely intracellular target of 4-quinolones is a DNA topoisomerase II-like enzyme located in mitochondria. This is supported by the work of Castora et al. (1983) in which they demonstrate that 4-quinolones inhibit mtDNA synthesis in isolated rat mitochondria. Also, Melendy and Ray (1989) have demonstrated that mitochondria in the trypanosomatid Crithidia fasciculata contain a type II DNA topoisomerase that is sensitive to the 4-quinolone, nalidixic acid.

Replication of mtDNA leads to the formation of two interlocked daughter molecules (Clayton, 1982; Berk and Clayton, 1976). Resolution of these molecules is important in order to initiate another round of mtDNA replication and for segregation of newly replicated mtDNA molecules to each daughter during cell division. In order to resolve these catenated rings of DNA, it is necessary to break the phosphodiester backbone of one of the mtDNA molecules, pass the second molecule's DNA through the break, and then reseal the break site. This function is thought to be mediated by a mitochondrial type II DNA topoisomerase activity. Whether or not this mitochondrial type II DNA topoisomerase is distinctly mitochondrial (different from the nuclear form) has been a question of much debate.

An enzyme that acts in two different cellular compartments is not unprecedented because several proteins are shared between the nuclear, cytosolic, and mitochondrial compartments. These proteins include tRNA modification enzymes, p21, cytosolic thyroid binding protein, thyroxine 5'-monodeiodinase, iodomelatonin binding sites, and RNase mitochondrial RNA processing (MRP) enzymes, and fumarase (Slusher et al., 1991; Ng et al., 1992; Yamaki et al., 1991; Yuan and Pang, 1991; Topper and Clayton, 1990; Suzuki et al., 1989).

Type II topoisomerases are enzymes that change the topology of DNA by introducing transient double-stranded breaks in DNA (Liu, 1989). These transient DNA breaks are unusual because they: 1) are a reversible; 2) occur at specific sites in DNA; and 3) involve protein linkage (of the enzyme's subunits) to the 5' ends of the DNA breaks. It was our goal to demonstrate, through the use of specific inhibitors that trap mtDNA-topoisomerase II enzyme intermediates, that mitochondria contained a type II DNA topoisomerase. This enzyme would be identified by analyzing the topoisomerase II enzyme-mtDNA cleavage products for the hallmarks stated above.

Results

One hallmark of type II topoisomerases is that they introduce transient double-stranded breaks in DNA. To determine if nalidixic acid interacted with a mitochondrial topoisomerase II enzyme, mtDNA from L1210 cells treated with nalidixic acid as analyzed for the presence of double-stranded breaks. Mouse L1210 cells in various phases of growth (early log, late log, and plateau phase) were treated with various concentrations of nalidixic acid for one hour and then lysed with buffer containing SDS. Total cellular DNA was isolated, restricted with Sac I (a restriction enzyme that cuts the mouse mtDNA once), and analyzed by electrophoresis and Southern blotting using the mouse mtDNA specific probe pMBS2.1 (Figure 5-1). There was no significant cleavage of mtDNA in untreated L1210 cells. However, nalidixic acid stimulated cleavage of mtDNA at concentrations between 5 and 25 μg/ml in cells maintained in early log phase growth (lanes C and D). Drug stimulated mtDNA cleavage occurred at multiple and specific sites in the mtDNA. Interestingly, nalidixic acid at 25 μg/ml (lane D) stimulated less cleavage than nalidixic acid at 5 μg/ml (lane C), suggesting an autoinhibitory effect on mtDNA cleavage. At lower drug concentrations, the religation step of the enzyme in inhibited, forming clevable-complexes that can be denatured into strand breaks. At higher drug concentrations, the enzyme's catalytic activity is almost completely inhibited, thereby preventing the enzyme from binding and cleaving the DNA at all. A similar autoinhibitory effect was seen with the mitochondrial type II topoisomerase identified in trypansomes (Shapiro and Englund, 1990) as well as with intercalating agents that target nuclear type II topoisomerase (Chen et al., 1984). In addition, at high concentrations, the 4-quinolones nalidixic acid and norfloxacin have been shown to unwind DNA, which may inhibit the topoisomerase II enzyme from binding or cleaving DNA because of changes in DNA structure (Tornaletti and Pedrini, 1988). Cleavage fragments stimulated by nalidixic acid were mapped by indirect end labeling and the locations are shown in Figure 5-7. Drug induced cleavage was not observed in late log or plateau phase growth, suggesting that cleavage was dependent on cell profileration.

Ciprofloxacin was also shown to stimulate cleavage of mtDNA in mouse L1210 cells (Figure 5-2). Mitochondrial DNA from mouse L1210 cells treated with various concentrations of ciprofloxacin was analyzed for the presence of

Figure 5-1 -Growth Phase Dependency of Nalidixic Acid induced Mitochondrial DNA Strand Breaks

Mouse L1210 cells were seeded at densities of 1.5 X 10^5 (early log), 5 X 10^5 (late log), and 2 X10^6 (plateau) cells/ml. Cells were exposed to 0 (lanes A, E, and H),1 (lane B),5 (lanes C, f, and I),and 25 (lanes D, G, and J) µg/ml nalidixic acid for 1 hr. After drug exposure, the cells were pelleted and total cellular DNA was isolated as described in MATERIALS AND METHODS. The samples were restricted with Sac I and electrophoresed for 18 hr at 70 volts in a 0.7% agarose gel. The mitochondrial DNA was analyzed by Southern blotting using the mitochondrial DNA subclone pMBS2.1 and is representative of 2 experiments. Arrow denotes position of linear mitochondrial DNA.

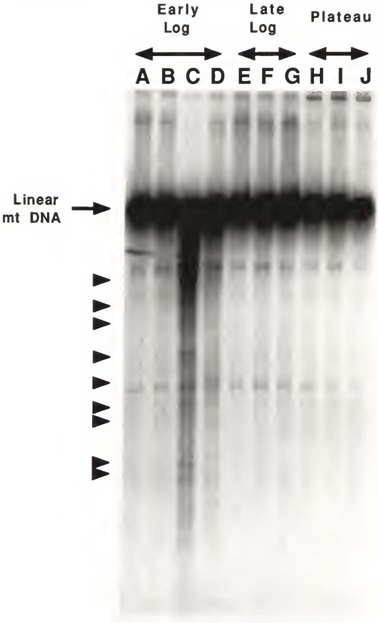
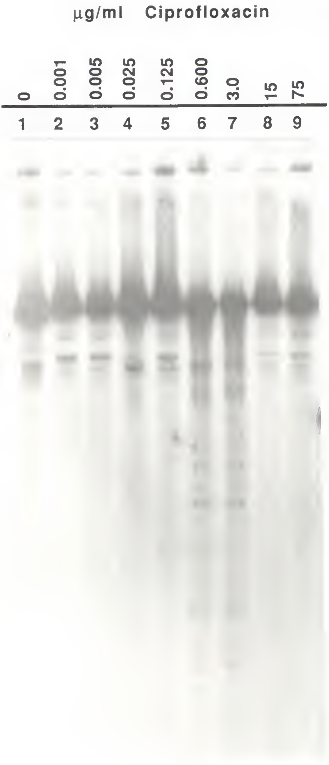

Early Log
Late Log
Plateau
A B C D E F G H I J
Linear mt DNA

Figure 5-2 -Ciprofloxacin Induces Mitochondrial DNA Strand Breaks

L1210 cells at a density of 1-2 X 10^5 cells/ml were exposed to various concentrations of ciprofloxacin for 1 hr before they were harvested and total cellular DNA purified as described in MATERIALS AND METHODS. After restricting the DNA with Sac I, the samples were electrophoresed through a 0.7% agarose gel for 18 hrs at 70 volts and Southern blotted using the mouse mitochondrial DNA subclone pMBS2.1 as the probe and is representative of 3 experiments. Lanes 1-9: 0, 0.001, 0.005, 0.025, 0.125, 0.600, 3.0, 15, 75 µg/ml ciprofloxacin.


µg/ml Ciprofloxacin
0
0.001
0.005
0.025
0.125
0.600
3.0
15
75
1
2
3
4
5
6
7
8
9

strand breaks as described above. Ciprofloxacin stimulated cleavage of mtDNA at concentrations between 0.6 and 3 μg/ml (lanes 6 and 7). No cleavage of mtDNA was detected at concentrations over 3 μg/ml (lanes 8 and 9) or at concentrations below 0.3 μg/ml (lanes 2-5), suggesting an autoinhibitory effect on DNA cleavage. Ciprofloxacin stimulated cleavage of mtDNA also occurred at specific sites of the mtDNA. Interestingly, all samples including the untreated L1210 cells (lane 1) contained several discrete bands slightly smaller than full length linear mtDNA, suggesting that some endogenous cleavage occurred even in the absence of drug. These mtDNA cleavage fragments, as well as those stimulated by ciprofloxacin, were mapped by indirect end labeling and the locations are shown in Figure 5-7.

Protein-linked DNA can be specifically bound to glass in the presence of 0.4 M guanidine and 0.3 M NaCl (Shin and Snapka, 1990). To deterimine if this assay would useful in detecting topoisomerase II-DNA complexes, mouse L1210 cells prelabeled in media containing ^{3}H-thymidine were incubated with various concentrations of VM-26, a nuclear topoisomerase II inhibitor. Cell lysates were then passed through glass fiber filters under conditions that specifically bound protein to glass. Filters were then counted to detect the presence of labeled DNA. DNA not bound to protein will not bind to glass under these conditions. VM-26 produced a dose dependent increase in protein-linked DNA formation (Figure 5-3). Protein-linked DNA from cells treated with 30 μM VM-26 no longer bound to the glass filters when the lysates were digested with proteinase K prior to filtering. Also, protein-linked DNA was not retained on glass filters after the filter was treated with proteinase K and washed a second time, suggesting that the DNA retained on the filter was protein linked. Nuclear topoisomerase II-DNA complexes are reversible upon heat treatment (Liu,

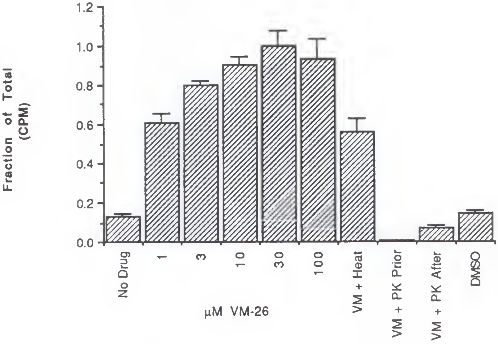


Figure 5-3 - Glass Binding of Protein Linked DNA from Cells Exposed to the Nuclear Topoisomerase II Inhibitor VM-26

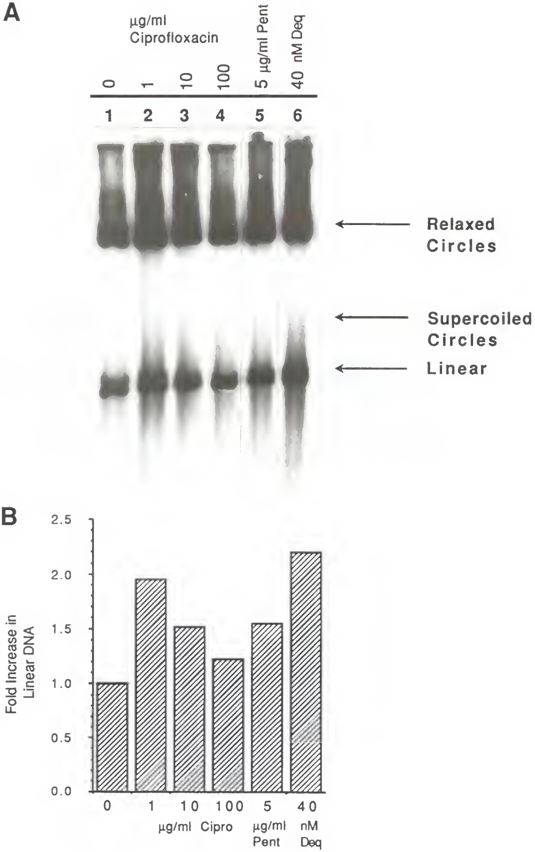
L1210 cells were labeled with 0.2 μC/ml ^{3}H-thymidine for 24 hrs before the cells were pelleted and resuspended in fresh label-free media and incubated for 2-3 hrs to remove unincorporated label. Equal numbers of cells were then incubated in 5 ml of fresh drug containing media for 1 hr before the cells were pelleted and the supernatant discarded. The samples were then lysed with 200 μl of 20 mM Tris pH 8.0, 1% SDS, 1 mM PMSF and passed through glass fiber filters under conditions that specifically bind protein (see MATERIALS AND METHODS). Samples were exposed to either 0, 1, 3, 10, 30, or 100 μM VM-26. Some samples treated with 30 μM VM-26 were either 1) exposed to a 65°C heat treatment for 10 min just prior to cell lysis, 2) subject to proteinase K (250 μg/ml) treatment for 30 min. (no PMSF added) before filtering, or 3) bound to glass filters and then treated with proteinase K (no PMSF) and washed a second time. Each sample was performed in triplicate and the data expressed as fraction of total counts ± standard error.

1989). Heat treatment of drug treated cells prior to lysis decreased the formation of protein-linked DNA by half, suggesting that DNA binding to the filter during VM-26 exposure was in fact due to the formation of nuclear topoisomerase II mediated cleavable-complexes. The protein covalently linked to the DNA strand breaks during VM-26 exposure has been previously been shown to be DNA topoisomerase II (Chen et al., 1984; Ross et al., 1984).

These results suggested the glass binding assay could be used to measure topoisomerase II-DNA cleavable-complex formation. Using a similar approach, it was investigated whether or not ciprofloxacin induced breaks in mtDNA that were protein-linked. HeLa cells, grown in a media in which glutamine was the sole energy source, were treated with ciprofloxacin (Figure 5-4). Two other drugs that deplete mtDNA from cells, pentamidine and dequalinium, were also analyzed for protein-linked mtDNA. Protein-linked DNA was isolated using the glass binding assay, except the DNA was bound to glass beads instead of glass fiber filters. After binding of protein-linked DNA, glass beads were treated with proteinase K before analysis by electrophoresis and Southern blotting using the human mtDNA specific probe pKB Mbo I 2.8. Untreated HeLa cells contained a small amount of mtDNA that bound to glass (lane 1), suggesting that a small amount of endogenous protein-linked mtDNA occured in the absence of drug. Ciprofloxacin at a concentration of 1 µg/ml stimulated protein-linked mtDNA formation by 2-fold (lane 2). Interestingly, higher concentrations of ciprofloxacin (lanes 3 and 4) did not futher stimulate protein-linked mtDNA formation, diplaying an autoinhibitory effect. Pentamidine at 5 µg/ml stimulated protein-linked mtDNA formation by 1.5-fold over untreated HeLa cells (lane 5). Dequalinium at a concentration of 40 nM stimulated protein-linked mtDNA formation by 2.3-fold increase over untreated HeLa cells

Figure 5-4 - Ciprofloxacin, Pentamidine, and Dequalinium Induce Protein-Linked Mitochondrial DNA in HeLa Cells

HeLa cells grown in media containing glutamine as the only energy source (Wice et al., 1981) were incubated at 37°C with ciprofloxacin, pentamidine, or dequalinium for 1.5 hrs before the samples were lysed and bound to glass beads as described in MATERIALS AND METHODS. Glass beads and elution buffer were then loaded into a well of a 0.7% agarose gel and electrophoresed for 18 hrs at 70 volts. The gel was then analyzed by Southern blotting using the Human mtDNA clone as a probe and is representative of 4 experiments preformed in vivo or in vitro.
(A) Autoradiogram lane 1- no drug; lanes 2, 3, and 4- 1, 10, 100 µg/ml ciprofloxacin; lane 5- 5 µg/ml pentamidine; lane 6- 40 mM dequalinium.
(B) Quantification of the linear band using a betascope 603 blot analyzer.

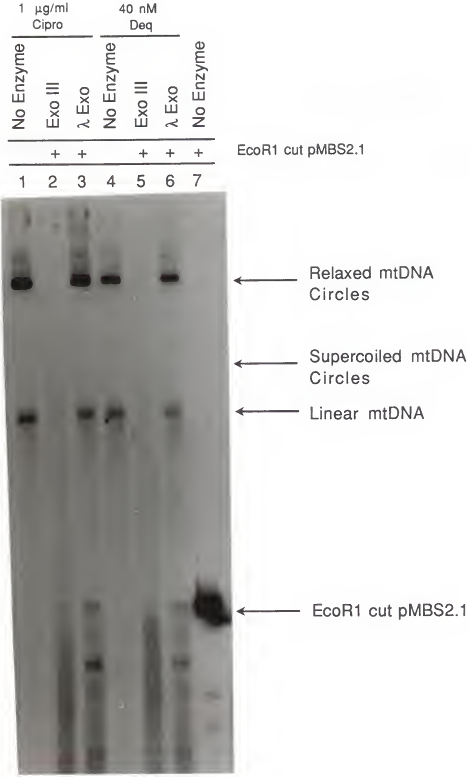

A
µg/ml
Ciprofloxacin
0
1
10
100
5 µg/ml Pent
40 nM Deq
1
2
3
4
5
6
Relaxed
Circles
Supercoiled
Circles
Linear
B
2.5
2.0
1.5
1.0
0.5
0.0
Fold Increase in
Linear DNA
0
1
10
100
5
40
µg/ml Cipro
µg/ml
Pent
nM
Deq

(lane 6). These results are consistent with the findings of Shapiro and Englund (1990) where they demonstrated that inhibitors of the trypansosomal mitochondrial type II topoisomerase produced a 2-fold increase in the formation of cleavage fragments during drug exposure. These authors also observed an autoinhibitory effect of these drugs on cleavable-complex formation. Interestingly, only relaxed and linear forms of mtDNA bound to glass. Nuclear type II and the trypanosomal mitochondrial type II topoisomerases as well as DNA gryase have also been shown to produce single-stranded DNA breaks in the presence of drugs (Liu, 1989; Shapiro and Englund, 1990; Shapiro et al., 1989; Gellert et al., 1977). These results suggested that ciprofloxacin, pentamidine, and dequalinium stimulate protein-linked breaks in mtDNA.

To examine the orientation of protein linkage, an exonuclease protection assay was performed on glass bound mtDNA from cells treated with either 1 μg/ml ciprofloxacin or 40 nM dequalinium (Figure 5-5). Protein-linked DNA was isolated as above by the glass binding assay after which glass beads were treated with proteinase K and DNA eluted. DNA was then purified and subject to various enzymatic treatments before analysis by electrophoresis and Southern blotting as above. Again, only relaxed and linear species of mtDNA bound to glass after treatment to 1 μg/ml ciprofloxacin or 40 nM dequalinium (lanes 1 and 4). DNA eluted from glass beads treated with a 3' specific exonuclease (exonuclease III-Richardson et al., 1964) degraded the mtDNA by greater than 95% (lanes 2 and 5), suggesting that the 3' ends of the DNA breaks were not blocked. However, only 20-30 % of the DNA treated with a 5' specific exonuclease (lambda exonuclease-Little, 1967) was degraded (lane 3 and 6), suggesting that the 5' ends of the mtDNA were protected. To insure that the exonucleases were active in each sample, 100 pg of Eco RI cut

Figure 5-5 -Glass Bound Mitochondrial DNA is Resistant to Lambda Exonuclease Digestion but Not Exonuclease III Digestion

HeLa cells grown in media containing glutamine as the only energy source (Wice et al., 1981) were exposed to either 1 µg/ml ciprofloxacin or 40 nM dequalinium for 1.5 hrs before the samples were lysed, bound to glass, and the eluate prepared for exonuclease treatment as described in MATERIALS AND METHODS. Lanes 1-3 contain equal amounts of glass eluted DNA from cells treated with 1 µg/ml ciprofloxacin; lanes 4-6 contain equal amounts of glass eluted DNA from cells treated with 40 nM dequalinium; lane 7 contains 100 pg EcoRI cut pMBS2.1 DNA. Lanes 1 and 4 contain glass eluted DNA only; lanes 2 and 5 contain glass eluted DNA, 100 pg EcoRI cut pMBS2.1, and 10 units of exonuclease III; lanes 3 and 6 contain glass eluted DNA, 100 pg EcoRI cut pMBS2.1, and 1 unit lambda exonuclease. Autoradiograph contains one sample set of those performed in triplicate. Arrows indicate migration position of supercoiled, relaxed, and linear mtDNA species as well as linear pMBS2.1.


1 µg/ml Cipro
40 nM Deq
No Enzyme
Exo III
λ Exo
No Enzyme
Exo III
λ Exo
No Enzyme
+ + + + +
EcoR1 cut pMBS2.1
1 2 3 4 5 6 7
Relaxed mtDNA Circles
Supercoiled mtDNA Circles
Linear mtDNA
EcoR1 cut pMBS2.1

pMBS2.1 was added to each sample. Greater than 95% of the pMBS2.1 DNA was degraded, suggesting that the exonucleases were active in all samples.

Ciprofloxacin was shown to stimulate cleavage of mtDNA in isolated mitochondria. Mitochondria isolated from mouse L1210 cells were subject to various treatments. After treatment, the mitochondria were lysed, DNA purified, restricted with Sac I (an enzyme that cuts mouse mtDNA once), and analyzed by electrophoresis and Southern blotting using the mouse mtDNA specific probe pMBS2.1 (Figure 5-6). In untreated mitochondria, most of the mtDNA signal resided in the band corresponding to full length linear mtDNA (panel A, lane 1). Mitochondria treated with 2 mM $MgCl_2$ contain discrete bands spaning from full length linear mtDNA to the bottom of the blot (Panel A, lane 2). When an aliquot of mitochondria treated with $MgCl_2$ was placed into a tube containing an excess of EDTA before lysis (panel A, lane 3), the cleaved mtDNA disappeared, suggesting that $MgCl_2$ stimulated cleavage is reversible. Over 75% of the total radioactivity was present in the intact or unit length linear band in untreated and EDTA reversed mitochondria. Conversely, less than 30% of the total radioactivity was present in the intact or unit length linear band in mitochondria treated with 2 mM $MgCl_2$.

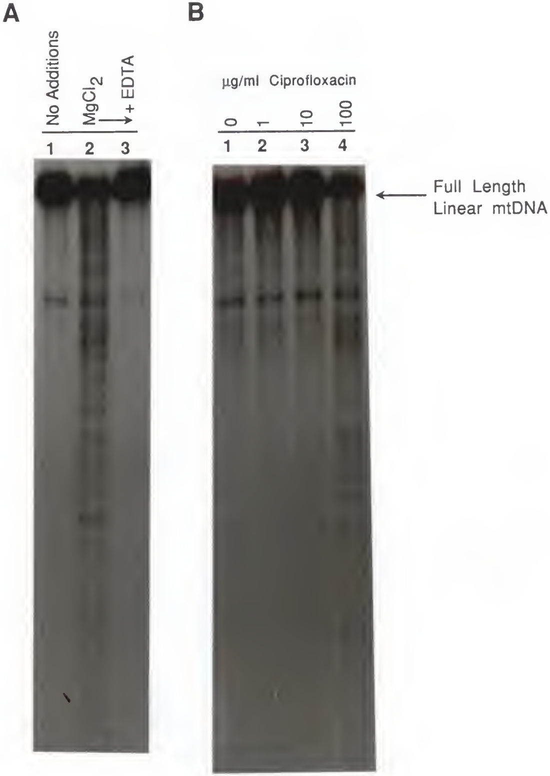
Ciprofloxacin at a concentration of 100 µg/ml stimulated cleavage of mtDNA in isolated mitochondria (Panel B, lane 4). No stimulation of mtDNA cleavage was observed during treatment of isolated mitochondria with concentrations of ciprofloxacin below 100 µg/ml (Panel B, lanes 1-3). Cleavage sites stimulated in mtDNA in isolated mitochondria were mapped by indirect end labeling and the locations are shown in Figure 5-7.

Figure 5-6 -Magnesium Chloride and Ciprofloxacin Stimulate Mitochondrial DNA Strand Breaks in Isolated Mitochondria

Mitochondria from 1 X 10^7 L1210 cells were isolated by differential centrifugation as described in MATERIALS AND METHODS. Mitochondria were resuspended in reaction buffer containing 1 mM ATP and 30 µl aliquots were subject to various treatments at 4°C. Mitochondria were then treated with various concentrations of $MgCl_2$ and/or ciprofloxacin. Any cleavable complexes formed were trapped by the addition of 1% SDS (final concentration) and the DNA purified asdescribed in MATERIALS AND METHODS. Samples were then restricted with Sac I electrophoresed for 18 hr at 70v in a 0.7% agarose gel and analyzed by Southern blotting using the mitochondrial DNA subclone pMBS2.1as described in MATERIALS AND METHODS. Magnesium stimulated cleavage was representative of 4 experiments, and ciprofloxacin stimulated cleavage was representative of 3 experiments.

(A) Autoradiogram of untreated samples, samples treated with $MgCl_2$, or samples treated with $MgCl_2$ and then treated with EDTA. Lane 1- untreated mitochondria in reaction buffer alone; lane 2- treated with 2 mM $MgCl_2$ for 10 min. at 4°C; lane 3- treated with 2 mM $MgCl_2$ for 10 min. at 4°C and then treated with 10 mM EDTA for 10 min. at 4°C.

(B) Autoradiogram of samples treated with various concentrations of ciprofloxacin for 10 min. at 4°C. All samples contain 0.5 mM $MgCl_2$. Lane1- 0.5 mM $MgCl_2$ alone; lane 2, 3, and 4- 1, 10, and 100 µg/ml ciprofloxacin respectively.


A
No Additions
MgCl$_2$
→ + EDTA
1
2
3
B
µg/ml Ciprofloxacin
0
1
10
100
1
2
3
4
Full Length
Linear mtDNA

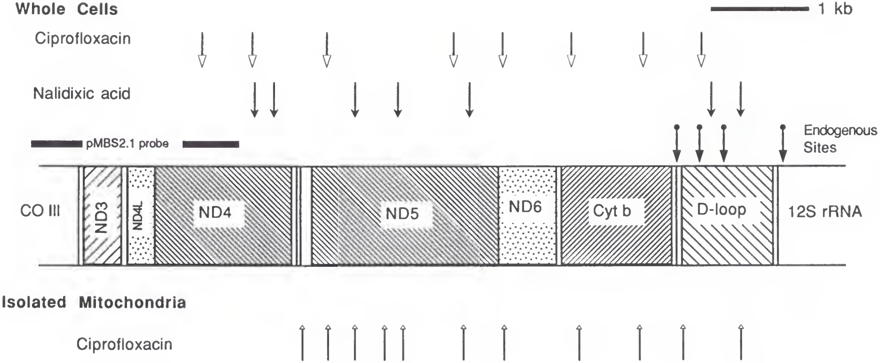


Figure 5-7 Map of Nalidixic Acid and Ciprofloxacin Induced mtDNA Cleavage Sites (see Figures 5-1, 5-2, and 5-7 for particular autoradiographs. see text for details)

Discussion

These results suggest that mitochondria contain an enzymatic activity that 1) stimulates reversible, double-stranded cleavage of mtDNA; 2) cleaves the mtDNA in specific sites; and 3) stimulates the linkage of protein to the 5' ends of the mtDNA breaks. These results are consistent with a type II and not a type I topoisomerase activity because the 5' ends and not the 3' ends of the mtDNA breaks were protein linked (Wang, 1985, Liu, 1989). This mitochondrial type II topoisomerase must exist inside mitochondria because protein was found linked directly to the 5' ends of the mtDNA. If this protein was not inside mitochondria, then it could not attach to the mtDNA, unless this protein attached to the mtDNA during or after lysis. Also, ciprofloxacin and dequalinium were shown to stimulate topoisomerase II mediated cleavage of mtDNA. These drugs do not affect the nuclear topoisomerase enzyme suggesting that this enzyme is pharmacologically distinct from the nuclear topoisomerase II enzyme.

A similar topoisomerase II-like activity was identified in isolated mitochondria. Visual inspection of the cleavage fragments produced by $MgCl_2$ and ciprofloxacin showed that many of the cleavage sites were found to be similar, suggesting that the mtDNA strand breaks may be mediated through the same enzyme. Doses required to produce cleavage in isolated mitochondria were much higher than that those needed to cleave the mtDNA in whole cells. Ciprofloxacin is concentrated inside cells at concentrations up to 10-15 times the extracellular concentration (Easmon and Crane, 1985). Therefore, concentrations required to stimulate cleavable complex formation upon mtDNA in whole cells would be 10- to 15-fold less. This topoisomerase II-like enzyme functions in the absence of the rest of the cell, suggesting that the eventual purification of this enzyme can be acheived. Isolated mitochondria may be an powerful system to study the activity and function of this type II topoisomerase. It may also prove to be an effective screen for potential new topoisomerase II inhibitors that are selective for the mitochondrial form.

A mammalian type II topoisomerase has not been purified to homogeneity. Castora et al. (1985) detected a decatenating activity in human acute leukemia cell mitochondria, but the stability limited complete characterization of this enzymatic activity. Recently, the same group has partially purified a DNA topoisomerase II activity from calf thymus mitochondria (Lin and Castora, 1991).

A mitochondrial type II topoisomerase has been purified to homogeneity in Crithidia fasciculata (Melendy and Ray, 1989). This enzyme had antigenic as well as biochemical differences from the nuclear type II topoisomerase (Melendy et al., 1988). Most interestingly, this enzyme was sensitive the 4-quinolone nalidixic acid. Also, a group has demonstrated that a drug sensitive type II topoisomerase enzyme exists in kinetoplasts of the trypanosomatid Trypanosoma equiperdum (Shapiro et al., 1989). This enzyme was shown to cleave minicircle kinetoplast DNA and produce DNA species with protein covalently attached to the 5' ends. Most interesting was the fact that this enzyme was sensitive to the anti-trypanosomal agents, berenil, pentamidine, and ethidium bromide. Nuclear topoisomerase II enzyme is unaffected by these agents, suggesting that this mitochondrial topoisomerase II was distinctly different from the nuclear form.

Many cleavage sites produced by nalidixic acid and ciprofloxacin were localized to the same regions of the mtDNA. Apparent differences in cleavage may be due to the doses of drug used; when the dose of nalidixic acid is increased to produce the same amount of strand breaks produced by ciprofloxacin, both ciprofloxacin and nalidixic acid may cleave at the same sites. This similarity of cleavage sites is not unprecedented, as it has been shown by other groups that topoisomerase-active compounds from the same drug class cause DNA cleavage at the same sites by nuclear type II topoisomerase (Tewey et al., 1984a). These results also suggest that mitochondria contain a topoisomerase II-like activity that is sensitive to the bacterial topoisomerase II (DNA gyrase) inhibitors, nalidixic acid and ciprofloxacin.

Some discrete bands appeared below the full length linear mtDNA band in non-drug treated samples. These cleavage sites, as well as some of the drug stimulated cleavage sites (see figures 5-1, 5-2, and 5-7), map within or very close to the D-loop (the origin of heavy strand replication), suggesting that this enzyme may play a role in the regulation of mtDNA replication. It has been shown in Chapter 3 of this dissertation that the drugs that affect the mitochondrial type II topoisomerase also cause a loss of mtDNA.

Type II topoisomerases are involved in many aspects of DNA and RNA metabolism such as 1) relieving torsional stress during transcription and DNA synthesis, 2) chromosomal condensation, 3) the segregation of daughter DNA molecules, and 4) DNA packaging in the nuclear scaffold (Nogguchi et al., 1983; Nelson et al., 1986; Ryoji et al., 1984; Earnshaw et al., 1985; Gasser et al., 1986). Mitochondrial type II DNA topoisomerases may play similar roles in the mitochondria as that of the DNA topoisomerase II in the nucleus. First, newly replicated mtDNA molecules must be segregated after replication. Also, the mitochondrial genome may have its own scaffolding structure. This is supported by the observation that most HeLa mtDNA molecules are associated with a proteinacous attachment to the mitochondrial inner membrane (Albring et al., 1977). Interestingly, this protein was located near the D-loop of the mtDNA, suggesting that this protein participates in a structural (and possibly a regulatory) role in mtDNA organization and replication. There is no evidence that this protein is a topoisomerase enzyme, but it may interact with a topoisomerase enzyme; recall that some endogenous cleavage sites are located within or near the origin of replication (D-loop).

Any enzyme that could affect the topological structure of mtDNA could affect the expression of mitochondrial encoded proteins. Barat-Gueride et al. (1989) demonstrated that the topological conformation of the DNA template modulated the activity of mitochondrial RNA polymerase in vitro. They found that partially and completely relaxed templates are much poorer substrates for transcription than are fully supercoiled substrates. Lansman and Clayton (1975) also have demonstrated that nicking of the mtDNA template in vivo, reduces transcriptional activity by 85%. Inhibition of type II topoisomerases in mammalian mitochondria would affect the topological state of mtDNA molecules, and thereby affecting the transcriptional efficiencies of these templates. Indeed, ciprofloxacin and dequalinium affect mitochondrial function (Lawrence et al., 1992; Weiss et al., 1987). Therefore, changes in mtDNA topology mediated by a type II DNA topoisomerase may affect the expression of mitochondrial genes that may lead to a loss of mitochondrial function. Future studies that assess the effects of the mitochondrial type II topoisomerase inhibitors on mitochondrial transcription should be performed.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Two antibacterial agents that target DNA gyrase (nalidixic acid and ciprofloxacin) were shown to deplete cells of their mtDNA. Associated with this loss of mtDNA, was a loss of mitochondrial encoded function. These effects were reversible upon drug removal. At concentrations that depleted cells of their mtDNA, ciprofloxacin was shown to be cytotoxic to cells only when drug exposures lasted over four cellular doublings, corresponding to a greater than 90% loss of the mtDNA. Cells grown in respiratory-independent conditions, by-passing the need for active mitochondria, were found to be resistant to the cytotoxic effects of ciprofloxacin. A likely molecular target for mtDNA depletion was identified as a type II DNA topoisomerase located in mitochondria. Evidence supporting this hypothesis came from studies demonstrating that ciprofloxacin induced the formation of protein linked mtDNA strand breaks that were 5' blocked. Other agents from several drug classes were also shown to deplete mtDNA from cells (ethidium bromide, Hoescht H33258, pentamidine, and dequalinium) and one of these drugs, dequalinium, has been shown to act through a mitochondrial type II DNA topoisomerase.

Our hypothesis of the molecular mechanism of ciprofloxacin induced cytotoxicity is as follows:

1. Ciprofloxacin inhibits the function of a mitochondrially located type II DNA topoisomerase, which is likely to play an important role in resolving topological problems that arise during mtDNA replication and transcription.

2. Nuclear DNA synthesis and cellular division continue unaffected, depleting the cellular mtDNA content in half at each successive mitotic event.
3. Eventually, the cell's mtDNA content drops below a 10%, a minimum level required for cells growth (Weisman and Attardi, 1987), producing a cytotoxic event.

This loss of mtDNA and cell viability are associated with concentrations of ciprofloxacin that produce an autoinhibitory effect on mtDNA cleavage. In contrast, nuclear topoisomerase mediated cytotoxicity requires the formation of cleavable-complexes. It is likely that inhibition of this enzyme may play an important role in mtDNA depletion, but future studies examining the effects of these drugs on the catalytic activity in vitro are necessary in order to resolve this issue.

These results raise an interesting question: is this enzyme unique from the nuclear form? Several enzymes are shared between the nuclear and mitochondrial conparments including tRNA modification enzymes, RNase mitochondrial RNA processing enzymes, and p21 (Slusher et al., 1991; topper and Clayton, 1990; Ng et al., 1992). VPM^{R}5 cells, a CHO cell line resistant to the nuclear topoisomerase II inhibitors through a mutation in the topoisomerase II gene, are sensitive to ciprofloxacin at the same concentrations that affect wild type CHO cells. Indeed, this piece of very preliminary evidence supports the existence of two distinct topoisomerase II enzymes within mammalian cells.

Future studies should include the purification and cloning of the mitochondrial type II DNA topoisomerase. Its purification will produce easier screening assays in the pursuit of more selective, highly potent pharmaceuticals directed at this enzyme. Also, the same assay would enable safety screens to be performed more rapidly, identifying potentially toxic agents early in the drug development process. Cloning this enzyme will aid in studies characterizing the location and expression of this gene. Also, selective antibodies raised against this enzyme will simplify studies aimed at examining the pre- and post-translational regulation. All in all, these future studies will aid in understanding the role these enzymes have in mitochondrial biogenesis, and correspondingly, their potential role as drug targets in chemotherapy.

Two major implications of this research impact drug development. First, the possibility of a new cancer chemotherapeutic target has been identified. Zinkewich-Peotti, et al. (1990) presented data suggesting that mtDNA is required to maintain a transformed phenotype in immortalized avian cells. If mtDNA is important in the mainatenance of cell growth phenotypes, such as cell transformation, then drugs that affect the mtDNA copy number may be useful chemotherapeutic agents. Ditercalinium and dequalinium, two agents that have been shown to affect mitochondrial function, have been shown to have significant anticarcinoma activity (Esnault et al., 1990; Weiss et al., 1987). Therefore, an enzyme that has an important role in mtDNA maintenance, such as a mitochodnrial type II DNA topoisomerase, may be a useful target in cancer chemotherapy. Ciprofloxacin and dequalinium, may provide useful models that allow chemists to design more selective mitochondrial type II DNA topoiosmerase inhibitors. Also, through structure activity analysis, more potent inhibitors of this mitochondrial enzyme may produce agents that have significant antitumor activity in humans. Second, unwanted toxic side effects of therapeutic agents may be produced by the same mechanism. Clearly, tissues contain discrete mtDNA copy numbers, suggesting some tissues may be more susceptible to these types of inhibitors (Veltri et al., 1990). Alternately, different tissues may turn over their mtDNA at different rates (Gross et al., 1969). These results suggest that inhibition of mtDNA synthesis may have dramatic consequences on cell function and viability. For example, adriamycin has severely limiting therapeutic use due to its cardiotoxicity. It has been postulated that adriamycin affects mitochondrial function, but most of these studies examined the direct effects of adriamycin on oxidative phosphorylation. The clinical features of adriamycin induced cardiotoxicity are produced over long periods of time and have an accumulative dose effect. In fact, there is a limit of the total cumulative dose of adriamycin that can be administered to a person, suggesting that this drug is retained in cells and has effects long after drug administration has been completed. Moreover, adriamycin kills dividing cells through the production of nuclear DNA topoisomerase II-DNA complexes (Tewey et al., 1984b). Adriamycin may have selectivity for a nuclear topoisomerase II enzyme, but it may also has significant mitochondrial topoisomerase II activity only seen after long periods of time in nondividing cells. This is supported by a report that suggested that adriamycin caused mtDNA strand breaks and interacted with mtDNA (Ellis et al., 1987). Therefore, adriamycin may target a mitochondrial regulatory protein, such as a mitochondnrial type II DNA topoisomerase. Recently, the anti-HIV agent AZT has been shown to deplete mtDNA from patients displaying neuromuscular complications. These neuromuscular complications have been associated with a destructive mitochondrial myopathy. The molecular mechanism of AZT induced mtDNA depletion is thought to occur through inhibition of the gamma DNA polymerase (Chen and Cheng, 1989). Although this drug does not interact with a topoisomerase in mitochondria, the consequences are the same, a loss of mtDNA and a loss of mitochondrial function. These results suggest that drug related delayed myopathies may be related to a mtDNA depleting mechanism.

It is interesting that genomic mtDNA alterations are associated with several degenerative diseases including myoclonic epilepsy, ragged-red fiber disease, Leber's hereditary optic neuropathy, Kearns-Sayre syndrome, and Pearson's marrow/pancreas syndrome (Wallace, 1992). Many of these diseases are associated with mtDNA heteroplasmy. These individuals contain two mtDNA genomes, one of which usually contains insertions or deletions. The slow drift from one population of mitochondria to the other during development is thought to cause the onset of these diseases. One interesting possibility is that these mutant mtDNA populations arise through recombination. A recent report characterizing the mtDNA deletions in patients exhibiting several of these degenerative diseases demonstrated that the flanking regions of the mtDNA deletions contained sequences that corresponded to target sequences for Drosophila topoisomerase II (Mita et al., 1990). Type II DNA topoisomerases have been implicated in recombination (Bae et al., 1988) and drugs that affect these enzymes may modulate the incidence of recombinogenic mtDNA mutations. These results suggests that a mitochondrial type II DNA topoisomerase may play a role in the formation of these diseases.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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